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(54) Title: INTERCELLULAR ADHESION MEDIATORS

(57) Abstract

The present invention relates to compositions and methods for reducing or controlling inflammation and for treating inflammatory disease processes and other pathological conditions mediated by intercellular adhesion. Compositions which selectively bind a selectin receptor and which have at least one selectin-binding moiety are provided by the present invention. The selectin-binding moieties have the general formula: R_1-Galβ1,4(Fucα1,3)GlcNAc-(R_2)a wherein R_1 is an oligosaccharide or R_3-R_4-C(CO$_2$H)-, wherein R_3 and R_4 are the same or different and are -H, -C1-C8 alkyl, -hydroxyl C1-C8 alkyl, -aryl C1-C8 alkyl, or -alkoxy C1-C8 alkyl; wherein R_2 is β1, 3Gal, α1, 2Man or α1, 6GalNac and a is 0 or 1.

INTERCELLULAR ADHESION MEDIATORS

FIELD OF THE INVENTION

The present invention relates to compositions and methods for reducing or controlling inflammation and for treating inflammatory disease processes and other pathological conditions mediated by intercellular adhesion.

BACKGROUND OF THE INVENTION

Vascular endothelial cells and blood platelets play key roles in a number of biological responses by selectively binding certain cells, for instance phagocytic leukocytes, in the blood stream. For example, endothelial cells preferentially bind monocytes and granulocytes prior to their migration through the blood vessel wall and into surrounding tissue in an inflammatory response. Certain inflammation-triggering compounds are known to act directly on the vascular endothelium to promote the adhesion of leukocytes to vessel walls. Cells then move through the walls and into areas of injury or infection. Cellular adhesion to vascular endothelium is also thought to be involved in tumor metastasis. Circulating cancer cells apparently take advantage of the body's normal inflammatory mechanisms and bind to areas of blood vessel walls where the endothelium is activated.

Blood platelets are also involved in similar responses. Platelets are known to become activated during the initiation of hemostasis and undergo major morphological, biochemical, and functional changes (e.g., rapid granule exocytosis, or degranulation), in which the platelet alpha granule membrane fuses with the external plasma membrane. As a result, new cell surface proteins become expressed that confer on the activated platelet new functions, such as the ability to bind both other activated platelets and other cells. Activated platelets are recruited into growing thrombi, or are cleared rapidly from the blood circulation. Activated platelets are

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known to bind to phagocytic leukocytes, including monocytes and
neutrophils. Examples of pathological and other biological
processes which are thought to be mediated by this process
include atherosclerosis, blood clotting and inflammation.

5   Recent work has revealed that specialized cell
surface receptors on endothelial cells and platelets,
designated endothelial leukocyte adhesion molecule-1 (ELAM-1)
and granule membrane protein-140 (GMP-140), respectively, are
involved in the recognition of various circulating cells by the
10   endothelium and platelets. These receptors are surface
glycoproteins with a lectin-like domain, a region with homology
to epidermal growth factor, and a region with homology to
complement regulatory proteins (see, Bevilacqua et al., Science
243:1160 (1989), which is incorporated herein by reference).
15   For example, ELAM-1 has been shown to mediate endothelial
leukocyte adhesion, which is the first step in many
inflammatory responses. Specifically, ELAM-1 binds human
neutrophils, monocytes, eosinophils, certain T-lymphocytes (N.
Graber et al., J. Immunol., 145:819 (1990)), NK cells, and the
20   promyelocytic cell line HL-60.

          The term "selectin" has been suggested for a general
class of receptors, which includes ELAM-1 and GMP-140, because
of their lectin-like domain and the selective nature of their
adhesive functions. These cell surface receptors are expressed
25.   on a variety of cells. GMP-140 (also known as PADGEM) is
present on the surface of platelets and endothelial cells,
where it mediates platelet-leukocyte and endothelium-leukocyte
interactions. Another member of the selectin class is the MEL-
14 antigen, and its human analog LAM-1, which are cell surface
30   receptors of lymphocytes, and act as lymph node homing
receptors. The exact nature of the ligand recognized by
selectin receptors remains unknown.

          Various other methods have been previously developed
to block the action of selectins and thus inhibit cellular
35   adhesion. For instance, the use of monoclonal antibodies
directed to ELAM-1 has been proposed as a method to inhibit
endothelial-leukocyte adhesion as a treatment for pathological
responses, such as inflammation. Endothelial interleukin-8 has

also been shown to be an inhibitor of leukocyte-endothelial
interactions.

With the elucidation of the ligand-receptor
interaction, it will be possible to develop highly specific,
5   efficient inhibitors of selectin-mediated cellular adhesion
which would be useful in therapeutic regimens. The ligand(s)
could also be used to target other pharmaceutical compounds,
such as anti-inflammatory agents or anti-oxidants, to the sites
of injury. To date, insufficient understanding of the
10   interaction of the ligand(s) and receptor molecules on the
respective cells has hindered these efforts. The present
invention fulfills these and other related needs.

SUMMARY OF THE INVENTION

15   Novel compositions which selectively bind a selectin
receptor and which have at least one selectin-binding moiety
are provided by the present invention. The selectin-binding
moieties have the general formula:

$$R_1-Gal\beta1,4(Fuc\alpha1,3)GlcNAc-(R_2)_a$$
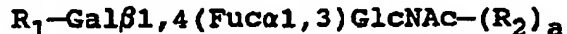
20   wherein R_1 is an oligosaccharide or $R_3-R_4-C(CO_2H)-$,
wherein R_3 and R_4 are the same or different and
are $-H$, $-$ C1-C8 alkyl, $-$ hydroxyl C1-C8 alkyl, $-$aryl
C1-C8 alkyl, or $-$alkoxy C1-C8 alkyl;
wherein R_2 is $\beta1,3Gal$, $\alpha1,2Man$, or $\alpha1,6GalNac$ and a
25   is 0 or 1.

In certain preferred embodiments, R_1 is a sialic
acid, usually NeuAc or NeuGc. If R_1 is an oligosaccharide it
will preferably be NeuAcα2,3Galβ1,4GlcNAcβ1,3 or
NeuGcα2,3Galβ1,4GlcNAcβ1,3. The selectin binding moieties are
30   typically oligosaccharides comprising the minimal
tetrasaccharide (termed SLX) recognized by the selectin
receptor.

The compound is typically prepared from a
polysaccharide having a repeat unit comprising the
35   unfucosylated SLX core structure. Upon fucosylation, a
polyvalent SLX-bearing polysaccharide is obtained. Preferred
polysaccharides for this purpose are type Ia, type II, and
type III, polysaccharides from Group B streptoccus. Polyvalent

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selectin-binding compounds are also obtained by linking
selectin-binding moieties to various linker moieties.

The claimed compositions inhibit intercellular
adhesion mediated by the selectin cell surface receptor and
5   thereby are capable, for example, of inhibiting inflammatory
and other pathological responses associated with cellular
adhesion. In related embodiments the composition that binds
the selectin may be a polysaccharide, a glycoprotein, a
glycolipid, or an oligosaccharide.

10   The present invention specifically provides the above
compounds in pharmaceutical compositions. The pharmaceutical
compositions can comprise, for example, liposomes which
comprise a moiety capable of selectively binding a selectin
receptor and a pharmaceutically acceptable carrier. The
15   liposome having the moiety may also serve as a targeting
vehicle for a conventional chemotherapeutic agent, which agent
is encapsulated within the liposome and delivered to targeted
cells which express a selectin receptor. Typically the
chemotherapeutic agent is an anti-inflammatory agent or an
20   anti-oxidant. Using the moieties described herein to target
chemical agents encapsulated within liposomes is a convenient
and effective method for reducing therapeutic levels of a drug
and minimizing side effects.

The pharmaceutical compositions of the present
25   invention may also comprise immunoglobulins capable of
selectively binding an oligosaccharide ligand recognized by a
selectin receptor. Suitable immunoglobulins for this purpose
include CSLEX-1, FH6, SNH_3, SNH_4 and VIM-2.

In other aspects, the invention comprises methods of
30   inhibiting intercellular adhesion in a patient for a disease
process, such as inflammation, by administering to the patient
a therapeutically effective dose of a compound comprising a
·moiety capable of binding a selectin receptor. The selectin
receptor, such as ELAM-1 or GMP-140, may be expressed on
35   vascular endothelial cells or platelets. The inflammatory
process may be, for example, septic shock, wound associated
sepsis, acute respiratory

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the ability of cells which express
SLX (LEC 11) to bind to IL-1β activated endothelial cells
compared to those cells which express non-sialylated Le^X (CHO-
5   K1 and LEC 12).

Fig. 2 illustrates the ability of monoclonal
antibodies specific for SLX to block selectin-mediated binding
of HL-60 cells at 37°C (Fig. 2A) and 4°C (Fig. 2B) compared to
monoclonal antibodies which do not bind SLX determinants.

10   Fig. 3 illustrates the effects of incubating LEC 11
(Fig. 3A) and LEC 12 (Fig. 3B) cells with SLX and non-SLX
specific monoclonal antibodies on binding to activated
endothelial cells.

Fig. 4 illustrates the results obtained by treating
15   HL-60, LEC11 and LEC12 cells with sialidase before binding to
activated endothelial cells.

Fig. 5 compares the ability of liposomes which
contain glycolipids with SLX, Le^X, or similar carbohydrate
structures to inhibit the binding of HL-60 cells to activated
20   endothelial cells.

Fig. 6 compares the inhibition of GMP-140 mediated
platelet adhesion by monoclonal antibodies specific for SLX and
Le^X determinants.

Fig. 7 compares the ability of liposomes which
25   contain glycolipids with SLX, Le^X, or similar carbohydrate
structures to inhibit the binding of HL-60 cells to activated
platelets.

Fig. 8 compares the ability of liposomes which
contain glycolipids with SLX, Le^X, or similar carbohydrate
30   structures to inhibit the binding of PMNs to activated
platelets.

Fig. 9 shows inhibition of GMP-140 mediated adhesion
by glycolipids with the terminal sialic acid either NeuAc or
NeuGc.

35   Fig. 10 shows prophylactically administered
monoclonal antibodies against ELAM-1 prevent lipopolysaccharide
induced death in rate.

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Fig. 11 shows therapeutically administered monoclonal antibodies against ELAM-1 prevent lipopolysaccharide induced death in rats.

5   DESCRIPTION OF THE PREFERRED EMBODIMENT
Compositions and methods are provided for inhibiting inflammatory and other disease responses mediated by cellular adhesion. The present invention also provides compounds (e.g., glycoconjugates and monoclonal antibodies) which have the
10   ability to block or inhibit the adhesion of the cells mediated by selectin cell surface receptors. Methods for preparing and screening for such compounds are also provided. Diagnostic and therapeutic uses for the compounds are provided.
A basis of the present invention is the discovery of
15   a carbohydrate moiety recognized by selectin cell surface receptors. As discussed above, selectins, also known as the "LEC-CAM" family of cell adhesion molecules, are unique glycoproteins expressed on the surface of a variety of cells. For instance, ELAM-1 is inducibly expressed on vascular
20   endothelial cells (Bevilacqua et al., supra and Hession et al., Proc. Nat'l. Acad. Sci., 87:1673-1677 (1990), both of which are incorporated herein by reference). This receptor has been demonstrated to be induced by inflammatory cytokines such as interleukin Iβ (IL-Iβ) and tumor necrosis factor α (TNFα), as
25   well as bacterial endotoxin (lipopolysaccharide) (see, Bevilacqua et al., Proc. Natl. Acad. Sci., 84:9238-9242 (1987) which is incorporated herein by reference). These compounds act directly on endothelial cells in vitro to substantially augment polymorphonuclear leukocyte (neutrophil), and monocyte
30   adhesion (Bevilacqua et al., Proc. Natl. Acad. Sci., supra).
As discussed above, GMP-140 is a membrane glycoprotein of platelet and endothelial secretory granules (Geng et al., Nature, 343, 757-760 (1990) which is incorporated herein by reference). Activated platelets which express GMP-
35   140 on their surface are known to bind to monocytes and neutrophils (Jungi et al., Blood 67:629-636 (1986)), and also to monocyte-like cell lines, e.g., HL60 and U937 (Jungi et al., supra; Silverstein et al., J. Clin. Invest. 79:867-874 (1987)),

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all of which are incorporated herein by reference. GMP-140 is
an alpha granule membrane protein of molecular weight 140,000
that is expressed on the surface of activated platelets upon
platelet stimulation and granule secretion (Hsu-Lin et al., J.
5   Biol. Chem. 259:9121-9126 (1984); Stenberg et al., J. Cell
Biol. 101:880-886 (1985); Berman et al., J. Clin. Invest.
78:130-137 (1986)). It is also found in megakaryocytes
(Beckstead et al., Blood 67:285-293 (1986)), and in endothelial
cells (McEver et al., Blood 70:355a (1987)) within the Weibel-
10 Palade bodies (Bonfanti et al., Blood 73:1109-1112 (1989)).
Furie et al. U.S. Patent No. 4,783,330, describe monoclonal
antibodies reactive with GPM-140. All of the foregoing
references are incorporated herein by reference.

        A third selectin receptor is the lymphocyte homing
15 receptor, MEL-14 antigen or LAM-1 (Gallatin et al., Nature
304:30-34 (1983); Siegellman et al., Science, 243:1165-1172
(1989); Rosen, Cell Biology, 1:913-919 (1989); and Lasky et al.
Cell 56:1045-1055 (1989) all of which are incorporated herein
by reference). In addition to lymphocyte homing, MEL-14
20 antigen/LAM-1 is believed to function early in neutrophil
binding to the endothelium.

        The structure and function of selectin receptors has
been elucidated by cloning and expression of full length cDNA
encoding each of the above receptors (see, e.g., Bevilacqua et
25 al., Science, supra, (ELAM-1), Geng et al., supra, (GMP 140),
and Lasky et al., supra, (MEL-14 antigen)). The extracellular
portion of selectins can be divided into three segments based
on homologies to previously described proteins. The N-terminal
region (about 120 amino acids) is related to the C-type
30 mammalian lectin protein family as described by Drickamer, J.
Biol. Chem., 263: 9557-9560 (1988) (which is incorporated
herein by reference) that includes low affinity IgE receptor
CD23. A polypeptide segment follows, which has a sequence that
is related to proteins containing the epidermal growth factor
35 (EGF) motif. Lastly, after the EGF domain are one or more
tandem repetitive motifs of about 60 amino acids each, related
to those found in a family of complement regulatory proteins.

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Since selectin receptors comprise a lectin-like
domain, the specificity of the molecules is likely to be based
on protein-carbohydrate interactions. Evidence provided here
indicates that a sialylated, fucosylated N-acetyllactosamine
5   unit of the Lewis X antigen, designated here as SLX, is a
moiety recognized by the lectin region of the selectin
receptor. In particular, the evidence shows recognition of
this moiety by both ELAM-1 and GMP-140. Compounds of the
present invention comprise this fucosylated, sialylated N-
10   acetyllactosamine unit in a variety of configurations.

Selective binding as used herein refers to specific
recognition by one molecule (typically referred to as a
receptor) of another molecule (typically referred to as a
ligand) by the spatial or polar organization of a determinant
15   site on the second molecule. Selective binding between the two
molecules occurs where affinity is sufficiently strong.
Binding affinity is typically represented by the affinity
constant (K_a) for equilibrium concentrations of associated and
disassociated configurations, i.e., $K_a = [R-L]/[R][L]$ where
20   [R], [L], and [R-L] are the concentrations at equilibrium of
the receptor (R), ligand (L) and receptor-ligand complex (R-L),
respectively.

The specific binding interactions of the receptor and
ligand molecules typically include reversible noncovalent
25   associations such as electrostatic attraction, Van der Waals
forces, and hydrogen bonds. See generally, Stryer,
Biochemistry (W.H. Freeman and Company, N.Y. 3rd Ed. 1988),
which is incorporated herein by reference. Examples of
selective binding interactions include antibody-antigen
30   recognition, enzyme-substrate recognition, and the like.

The nomenclature used to describe the oligosaccharide
moieties of the present invention follows the conventional
· nomenclature. Standard abbreviations for individual
monosaccharides are used. For instance, 2-N-acetylglucosamine
35   is represented by GlcNAc, fucose is Fuc, galactose is Gal, and
glucose is Glc. Two sialic acids which may be present on the
oligosaccharides of the present invention are 5-N-
acetylneuraminic acid (NeuAc) and 5-N-glycolylneuraminic acid

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(NeuGc). Unless otherwise indicated, all sugars except fucose
(L-isomer) are D-isomers in the cyclic configuration (e.g.,
pyranose or furanose). The two anomers of the cyclic forms are
represented by α and β.

5   The monosaccharides are generally linked by
glycosidic bonds to form oligo- and polysaccharides. The
orientation of the bond with respect to the plane of the rings
is indicated by α and β. The particular carbon atoms that form
the bond between the two monosaccharides are also noted. Thus,

10   a β glycosidic bond between C-1 of galactose and C-4 of glucose
is represented by Galβ1,4Glc. For the D-sugars (e.g., D-
GlcNAc, D-Gal, and D-NeuAc) the designation α means the
hydroxyl attached to C-1 (C-2 in NeuAc) is below the plane of
the ring and β is above the ring. In the case of L-fucose, the

15   α designation means the hydroxyl is above the ring and β means
it is below.

          Having identified SLX as a carbohydrate ligand that
mediates leukocyte-endothelial and leukocyte-platelet cell
adhesion, compounds comprising SLX and related structures can

20   be purified or synthesized de novo. As detailed below, the
present invention provides a variety of compounds comprising
the selectin-binding moieties of the present invention. For
instance, biomolecules can be used as the moiety-bearing
compound. Biomolecules as defined here include but are not

25   limited to biologically significant molecules such as amino
acids (and their mimetics), oligopeptides, proteins (e.g.,
glycoproteins and protein hormones), fatty acids, lipids (e.g.,
glycolipids, phospholipids, sphingolipids and gangliosides),
steroid hormones, oligosaccharides, polysaccharides, and

30   nucleic acids (e.g., deoxyribonucleic acids and ribonucleic
acids). These compounds can be purified and/or synthesized
according to standard techniques known to the skilled artisan.
In addition, a wide variety of compounds bearing the moiety may
be synthesized de novo as described below.

35   Once obtained, such compounds can be used for a
variety of purposes, including, for example, competitive
inhibition of the binding of SLX-bearing cells to cells which
express the selectin receptors. By binding of the compounds of

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the invention to a cell surface selectin, interaction of the
selectin with the native SLX ligand on migrating cells will be
prevented, interfering with normal and pathological binding of
leukocytes and other cells to the endothelium or platelets.

5   Thus, compounds which contain one or more selectin-binding
moieties can serve as effective inhibitors of, for instance,
inflammation, atherosclerosis, clotting and other endothelial
or platelet-mediated pathologies.

        The sialic acid residue in SLX may be in different
10   forms, so long as selectin binding is not significantly
affected. Typically, the sialic acid is 5-N-acetylneuraminic
acid, (NeuAc) or 5-N-glcolylneuraminic acid (NeuGc). Other
sialic acids may be used in their place, however. For a review
of different forms of sialic acid suitable in the present
15   invention see generally, R. Schauer, Methods in Enzymology, 50:
64-89 (1987), and Schaur, Advances in Carbohydrate Chemistry
and Biochemistry, 40: 131-234; both of which are incorporated
by reference. As demonstrated in Example IX, below, the
affinity for selectin receptors is the same if the
20   oligosaccharide terminates in NeuAc or NeuGc. Thus, the term
"SLX" as used herein refers to the minimal tetrasaccharide unit
(sialic acidα2,3Galβ1,4[Fucα1,3]GlcNA(β1,3) in which the sialic
acid is NeuAc, NeuGc or other equivalent forms of sialic acid.
Structures illustrated herein which show the sialic acid
25   residue as NeuAc are understood to include these other forms,
in particular NeuGc.

        Evidence provided below shows that a pentasaccharide
comprising the formula:
NeuAcα2,3Galβ1,4[Fucα1,3]GlcNAcβ1,3Galβ- is a minimal structure
30   having substantially more inhibitory effect than the
tetrasaccharide. Thus, in certain preferred embodiments the
oligosaccharide will comprise this pentasaccharide structure.

        Other variations on the basic SLX unit are also
recognized by selectin receptors. For instance, evidence
35   provided in Example VIII, below, shows that an oligosaccharide
moiety, termed SY2 (also known as the VIM-2 antigen), having
the structure
NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ

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binds selectin receptors equally as well as SLX. The SY2
moiety comprises two sialylated N-acetyllactosamine units, one
of which is SLX. Thus, oligosaccharides recognized by selectin
receptors may comprise a number of the sialylated N-

5   acetyllactosamine units, at least one of which is fucosylated
(see, Teimeyer et al., Proc. Natl. Acad. Sci. (USA) 88:1138-
1142 (1991), which is incorporated herein by reference.

The oligosaccharide moiety of the present invention
preferably terminates in a sialic acid residue. In certain

10   embodiments the sialic acid residue can be further linked to
other saccharide residues, such as a second sialic acid in an
α2,8 linkage.

Alternatively, the terminal sialic acid residue may
be replaced by a variety of radicals. Thus, certain selectin

15   binding moieties of the present invention have the general
formula: R_1-NeuAcα2,3Galβ1,4GlcNAcβ1-,
wherein R_1 is $R_2R_3C(CO_2H)$-,
wherein R_2 and R_3 are the same or different and are H, lower
alkyl (C1-C8), hydroxyl lower alkyl (C1-C8), arylalkyl,

20   alkoxylalkyl. In addition, R_2 and R_3 may be connected to form
a 4-8 membered carbocyclic or heterocyclic ring.

Compounds containing SLX and related structures can
be obtained from the cell surface glycoproteins or glycolipids
from a number of cells. For instance, the SLX antigen is

25   present on N-linked carbohydrate groups of the cell surface
glycoproteins of LEC11 cells, a glycosylation mutant of CHO
cells. LEC11 expresses this unique glycopeptide which contains
a terminal structure bearing both sialic acid and fucose in the
SLX sequence:

30   NeuAcα2,3Galβ1,4GlcNAcβ1-R
                              |α1,3
                              Fuc

· where R is:

         2Man   Fuc
35 |α1,6   ±|α1,6
         Manβ1,4GlcNAcβ1,4GlcNAcβ1,Asn
           |α1,4
(±SLX)β1,2Man

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(See, Stanley et al., J. Biol. Chem., 263:11374 (1988), which
is incorporated herein by reference.) Using the procedure
described below, it was demonstrated that the LEC11 mutant
bound to activated human vascular endothelial cells. Neither
5   wild type CHO cells nor other related glycosylation mutant CHO
cell lines without the particular glycosylation pattern (SLX)
showed the same level of binding.

       Other sources that can be used to obtain the SLX unit
include any cell which naturally expresses the moiety on
10 glycolipid or glycoprotein carbohydrate groups. Thus,
polymorphonuclear neutrophils, lymphocytes, tumor cells or HL-
60 cells have been used to purify this unit. Other cells which
bind to activated vascular endothelium can also be used to
isolate the ligand (see, Symington et al., J. Immunol.
15 134:2498-2506 (1985), Mizoguchi et al., J. Biol. Chem.
259:11949-11957 (1984), Mizoguchi et al., J. Biol. Chem.
259:11943-11948 (1984), Paietta et al., Cancer Res. 48:28-287
(1988), all of which are incorporated herein by reference).

       Compounds containing SLX or its mimetics can be
20 prepared from natural sources using methods well known in the
art for isolating surface glycoproteins, glycopeptides,
oligosaccharides and glycolipids from cells (See, e.g., Gerard,
"Purification of glycoproteins" and Thomas et al.,
"Purification of membrane proteins," both in Guide to Protein
25 Purification, Vol. 182, Methods in Enzymology (Deutscher ed.,
1990), which is incorporated herein by reference). For
example, LEC11 cells can be used to obtain glycoprotein or
glycolipid which contains the SLX unit using, for instance, the
method described in Stanley et al., supra. Briefly, LEC11
30 cells are infected with vesicular stomatitis virus. The
structural carbohydrate alterations exhibited by LEC11 are then
expressed on the N-linked biantennary carbohydrates of the G
glycoprotein of the virus. The virus is purified by
equilibrium gradient centrifugation, and glycopeptides are
35 purified using proteinase digestion as described by Stanley et
al.

       Several approaches are used to isolate a selectin-
binding moiety from HL-60, HT-29, colo 205, neutrophils, and

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other cell lines which contain a ligand recognized by
selectins. Since the ligand is generally expressed on the cell
surface of these cell types, one approach consists of isolating
a plasma membrane fraction enriched in the ligand. Once plasma
5   membranes have been isolated, the ligands may be isolated and
subsequently identified using monoclonal antibodies,
particularly those which are reactive with the SLX
oligosaccharide and related structures, such as monoclonal
antibodies FH6, SNH3 and CSLEX-1.
10   To characterize a selectin ligand on a glycoprotein,
release of the oligosaccharide is generally the first step in
the structural analysis of the oligosaccharide chain. This is
accomplished by chemical cleavage of the protein-carbohydrate
linkage, or by specifically releasing the oligosaccharide with
15 endoglycosidases. In most cases, different procedures may be
used to establish the correct conditions for an individual
glycoprotein. Asparagine-linked oligosaccharides are released
by hydrazinolysis, endoglycosidases, vigorous alkaline
hydrolysis, and trifluoroacetolysis. O-linked carbohydrate
20 units are released by alkaline β-elimination. The
oligosaccharides are separated from the glycopeptides by gel
filtration. The resulting oligosaccharides are then separated
from each other using a combination of gel filtration, HPLC,
thin layer chromatography, and ion exchange chromatography.
25 The isolated oligosaccharides are then fully analyzed.
Complete structural analysis of the purified oligosaccharide
units requires the determination of the monosaccharide units,
their ring form, configuration (D or L), anomeric linkage (α or
β), the positions of the linkages between the sugars and their
30 sequence. In addition, the position of any substituent groups
are established. Methylation analysis is used to determine the
positions of the glycosidic linkages between the
monosaccharides. The anomeric configuration of the sugar
residues can be addressed using 500-MHz^{1}H NMR spectroscopy.
35 The conditions and methods used to perform a complete
structural carbohydrate analysis are described generally in
Beeley, Laboratory Techniques in Biochemistry and Molecular

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<u>Biology</u>, eds. Burdon and Knippenberg, Elsevier, Amsterdam
(1985), incorporated herein by reference.

 The state of the art techniques to fully characterize
the sugars of an oligosaccharide include the use of several

5 analytical techniques such as FAB-MS (fast atom bombardment-
mass spectrometry), HPAE (high pH anion exchange
chromatography) and ^{1}H-NMR. These techniques are
complementary. Recent examples of how these techniques are
used to fully characterize the structure of an oligosaccharide

10 can be found in the analysis by Spellman et al., <u>J. Biol. Chem</u>.
264:14100 (1989), and Stanley <u>et al</u>., <u>supra</u>. Other methods
include positive ion fast atom bombardment mass spectroscopy
(FAB-MS) and methylation analysis by gas chromatography –
electron impact mass spectroscopy (GC/EI-MS) (<u>see</u>, EPO

15 Application No. 89305153.2, which is incorporated herein by
reference).

 One approach to characterizing the selectin ligand on
glycolipids consists of disrupting the cells using organic
solvents, isolating the glycolipids, and identifying those

20 glycolipids reactive with monoclonal antibodies to SLX, such as
FH6, SNH3, SNH4, CSLEX-1, or VIM-2, for example, and then
determining the structure of the oligosaccharide chains. To
obtain glycolipids and gangliosides which contain SLX, standard
methods for glycolipid preparation can be used (<u>see</u>, <u>e.g.</u>,

25 Ledeen <u>et al</u>., <u>J. Neurochem</u>. 21:829 (1973), which is
incorporated herein by reference). For example, glycolipids
are extracted from HL-60, HT-29, PMNs, human leukocytes, and
other cell lines expressing the selectin ligand by methods
generally known to those skilled in the arts (<u>see</u>, e.g.,

30 Symington et al., <u>J. Immunol</u>. 134:2498 (1985) and Macher and
Beckstead, <u>Leukemia Res</u>. 14:119-130 (1990), which are
incorporated herein by reference). Cells are grown in
·suspension and are harvested by centrifugation. Glycolipids
are extracted from the cell pellet by chloroform/methanol 2:1

35 and isopropyl alcohol/hexane/water 55:25:20 as described by
Kannagi et al., <u>J. Biol. Chem</u>. 257:14865 (1982), which is
incorporated herein by reference. The resulting extracts are
partitioned by a chloroform/methanol/water (3:2:1) Folch

15

partition. The resulting upper phase of the extraction
contains gangliosides and the lower phase contains glycolipids.
The upper phase containing gangliosides
(glycosphingolipids that contain at least one sialic acid
5   moiety) are isolated and separated into neutral and acidic
fractions using DEAE-Sephadex chromatography as described in
detail by Ledeen and Yu, Methods Enzymol. 83:139 (1982), which
is incorporated herein by reference. The resulting
gangliosides are pooled, lyophilized, and dissolved in
10   chloroform/methanol (2:1). The lower phase of the Folch
partition contains glycolipids. These are isolated and
separated on preparative thin-layer chromatography using
chloroform/methanol/water (60:35:8) as the solvent system as
described by Symington.
15   To identify those gangliosides and glycolipids which
contain a selectin ligand, immunochemical glycolipid analysis
is performed according to the procedure of Magnani et al.,
Anal. Biochem. 109:399 (1980), which is incorporated herein by
reference. Briefly, the ganglioside pool described above is
20   chromatographed by thin layer chromatography. The thin layer
plate is then incubated with ^{125}I labeled CSLEX-1, or other
monoclonal antibody which binds specifically to SLX or related
structures. Following incubation with the labeled antibody,
the plate is exposed to radiographic detection film and
25   developed. Black spots on the X-ray film correspond to
gangliosides that bind to the monoclonal antibody, and those
gangliosides are recovered by scraping the corresponding areas
of the silica plate and eluting the gangliosides with
chloroform/methanol/water. Glycolipids are also dried and
30   resuspended in chloroform and developed in a similar thin layer
system and probed with the radiolabeled antibody. Structural
analysis of oligosaccharides derived from glycolipids is
performed essentially as described for glycoproteins.
Oligosaccharides comprising the SLX unit can be
35   prepared from glycoproteins by methods well known in the art
(see, e.g., Gerard, supra, at pp. 537-539). Typically,
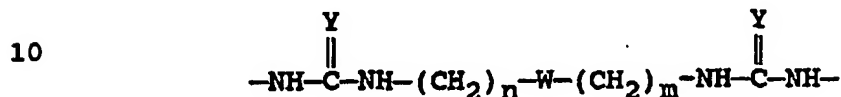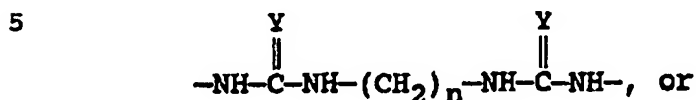N-glycosidase F (N-glycanase) is used to cleave N-linked

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oligosaccharides while O-linked groups are cleaved with endo-N-acetylgalactosaminidase.

Synthetic compounds containing SLX or its mimetics attached to a variety of moieties can be prepared depending on the particular use desired. For example, SLX can be converted to a ganglioside by linking a ceramide moiety to the C-1 of the reducing terminal GlcNAc unit. SLX structures and related structures can also be linked to a wide variety of other moieties such as variously substituted amino groups, heterocyclic compounds, ether linkages with branched or unbranched carbon chains, and ether linkages with aryl or alkylaryl moieties. The selectin-binding moiety may also be bound to various polysaccharides, amino acids, amino acid mimetics, oligopeptides or proteins using methods well known in the art.

The term "alkyl" as used herein means a branched or unbranched saturated or unsaturated hydrocarbon chain, including lower alkyls of 1-8 carbons such as methyl, ethyl, n-propyl, butyl, n-hexyl, and the like, cycloalkyls (3-7 carbons), cycloalkylmethyls (4-8 carbons), and arylalkyls. The term "aryl" refers to a radical derived from an aromatic hydrocarbon by the removal of one atom, e.g., phenyl from benzene. The aromatic hydrocarbon may have more than one unsaturated carbon ring, e.g., naphthyl. The term "alkoxyl" refers to alkyl radicals attached to the remainder of the molecule by an oxygen, e.g., ethoxyl. The term "heterocyclic compounds" refers to ring compounds having three or more atoms in which at least one of the atoms is other than carbon (e.g., N, O, S, Se, P, or As). Examples of such compounds include furans, pyrimidines, purines, pyrazines and the like. The term "oligo" refers to a polymeric molecule consisting of 2 to approximately 10 residues, for example, amino acids (oligopeptide), monosaccharides(oligosaccharide), and nucleic acids (oligonucleotide). The term "poly" refers to a polymeric molecule comprising greater than 10 residues.

For the synthesis of polyvalent forms of selectin-binding moieties, monomeric units containing SLX or other structures can be joined to form molecules having one to about

four or more selectin-binding moieties. An example of such a
polyvalent form is one in which the units are linked by the
following linking moieties:

$$-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-(CH_2)_n-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-, \text{ or}$$

$$-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-(CH_2)_n-W-(CH_2)_m-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-$$

wherein, n and m are the same or different and are integers
from 2 to 12; Y is O or S; and W is O, S, or NH.
Alternatively,
the moiety is a 5- to 14-membered ring having two substituents,
each substituent having the formula

$$-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-$$

wherein, Y is O or S, the substituents being in a cis- or
trans-relationship.

        SLX and related structures may also be attached to
various a heterocyclic compounds (e.g., one comprising nitrogen
atoms). In this case, the moieties are preferably linked to
the nitrogen atoms on the ring, each nitrogen being linked to
one moiety. Examples of heterocyclic compounds that are
suitable for this purpose include piperazine and
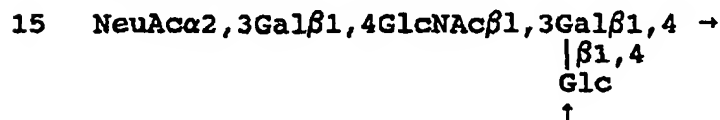homopiperazine.

        Alternatively, polyvalent forms of SLX or its
mimetics can be created by attaching the desired moiety to
preformed carrier moieties with multiple sites of attachment.
Examples include attachment of SLX to the amino groups of
lysine and lysine-containing peptides, proteins, glycoproteins
or the asparagine side-chain of such compounds.

        Another method of preparing polyvalent selectin-
binding compounds is by addition of desired monosaccharide
residues to polysaccharides. For instance, a polysaccharide
which contains a repeat unit having the linear core structure
of SLX (i.e, without the fucose residue) may be converted into
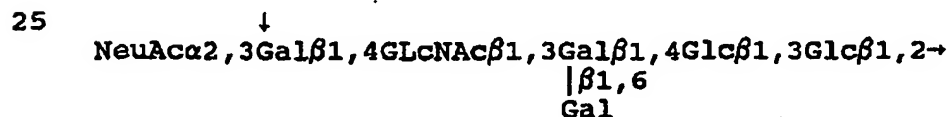a polyvalent SLX containing polysaccharide by enzymatic

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fucosylation. Native polysaccharide types Ia, II, or III
obtained from Group B Streptococcus are preferably used for
this purpose. These polysaccharides can be isolated according
to standard procedures from cell lines deposited with the
5   American Type Culture Collection (Type Ia from ATCC Nos. 12400
and 31574; Type II from ATCC Nos. 12973 and 31576; and Type III
from ATCC No. 31577). See e.g., Jennings et al., Biochem. 22
1258-1263 (1983) and PCT Application, Publication No. 8706267,
both of which are incorporated herein by reference.
10   These polysaccharides comprises repeat units having
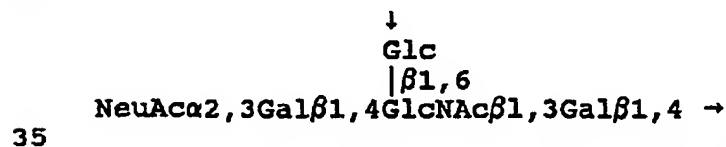the formulas:

Type Ia

15   NeuAcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4 →
                                    |β1,4
                                    Glc
                                    ↑

20

Type II

25   ↓
     NeuAcα2,3Galβ1,4GLcNAcβ1,3Galβ1,4Glcβ1,3Glcβ1,2→
                                    |β1,6
                                    Gal
     Type III
30
                      ↓
                      Glc
                      |β1,6
     NeuAcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4 →
35
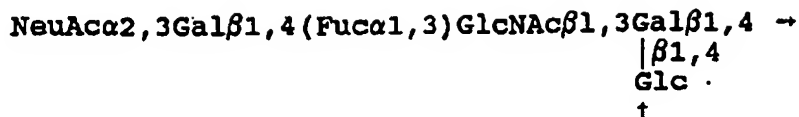   The arrows in the above structures identify the
backbone in each polysaccharide molecule. As can be seen, type
Ia polysaccharides contain a repeat unit having side chain
which corresponds to the SLX linear core structure. The other
40   two polysaccharides, the backbone comprises the SLX core
·structure. Enzymatic fucosylation of these polysaccharides
using an α1,3 fucosyltransferase according to standard
techniques described below yields a polyvalent SLX compound.
After fucosylation, the repeat units have the following
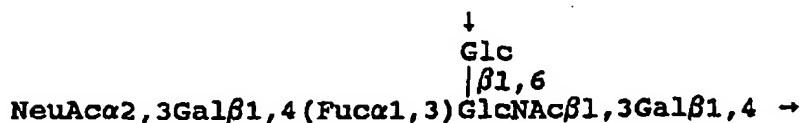45   formulas:

Type Ia

NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4 →
                                         |β1,4
5   Glc ·
                                         ↑

10
Type II

                ↓
NeuAcα2,3Galβ1,4(Fucα1,3)GLcNAcβ1,3Galβ1,4Glcβ1,3Glcβ1,2→
15 |β1,6
                                          Gal
Type III

                              ↓
20   Glc
                              |β1,6
NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4 →

25   The entire polysaccharide can be used for this
purpose as well as fragments thereof. Thus, polysaccharides
having a molecular weight between about 5,000 and about 300,000
can be used. A molecular weight between about 25,000 and about
100,000 is preferred. Any number of side chains on the
30   polysaccharide type Ia may be fucosylated for the
polysaccharide to have activity. Typically, between about 5
and about 200 side chains are fucosylated, preferably between
about 50 and about 150 are fucosylated.
            The synthesis of the selectin-binding moiety can be
35   accomplished using chemical, enzymatic, or combined chemical
and enzymatic strategies. (see, e.g., EPO Publication No.
319,253, which is incorporated herein by reference.) In a
preferred method (Scheme I below), a compound containing one or
more N-acetylglucosamine units (GlcNAc-R) can be reacted
40   sequentially with a galactosyltransferase (N-acetylglucosamine
· β1,4 galactosyltransferase (E.C. 2.4.1.90)), a sialytransferase
(Galβ1,4GlcNAc α2,3 sialyltransferase (E.C. 2.4.99.6) or
Galβ1,3GalNAc α2,3 sialytransferase (E.C. 2.4.99.4) and a
fucosyltransferase (N-acetylglucosaminide α1,3
45   fucosyltransferase (E.C. 2.4.1.152)) to yield the final SLX-
containing structures. In this case, R may be a carrier moiety

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or activatable intermediate that will allow attachment to a
suitable carrier moiety. Each enzymatic reaction uses the
appropriate nucleotide sugar as a donor substrate to generate
the following intermediates in the synthesis of SLX. The
5   glycosyl transfer reactions may optimally be carried out with
added alkaline phosphatase (e.g., from calf intestine, CIAP) to
consume the nucleoside phosphate byproduct which may inhibit
the reaction.

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Scheme 1
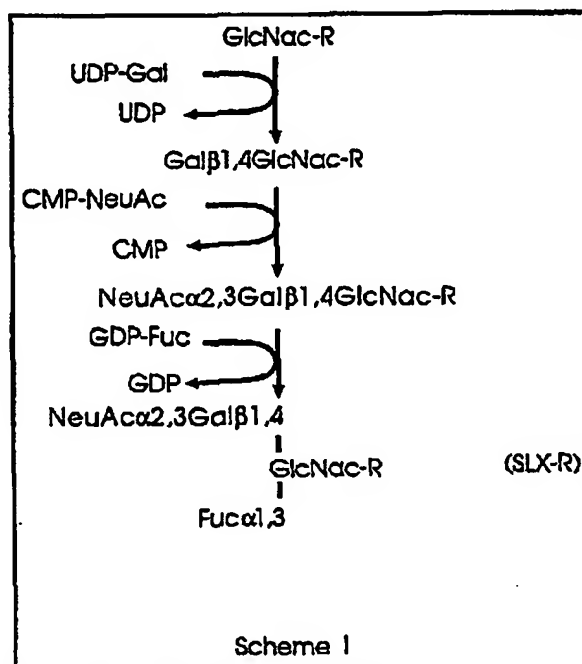
25   The general conditions for preparative enzymatic
synthesis of carbohydrate groups analogous to SLX are known
(see, e.g., Toone et al., Tetrahedron 45:5365-5422 (1989); Wong
et al., J. Am. Chem. Soc. 47:5416-5418 (1982); Unverzagt et
al., J. Am. Chem. Soc. 112:9308-9309 (1990); Prieels et al., J.
30 Biol. Chem. 256:10456-10463 (1981), all of which are
incorporated herein by reference). Each of the key enzymatic
reactions has been demonstrated (Beyer et al., Adv. Enzymol.
·52:23-176 (1981); Toone et al., supra; and Howard et al., J.
Biol. Chem. 262:16830-16837 (1981); all of which are
35 incorporated herein by reference). For preparative reactions,
the galactosyltransferase and the sialyltransferase(s) are
purified from natural sources (Beyer et al., supra, and
Weinstein et al., J. Biol. Chem. 257:13835-13844 (1982), which

are incorporated herein by reference). Fucosyltransferases may
also be identified from natural sources, as generally described
in Crawley and Hindsgaul, Carbohyd. Res. 193:249-256 (1989),
incorporated by reference herein. The cDNAs of the

5 galactosyltransferase and a sialyltransferase have been cloned
(Paulson and Colley, J. Biol.Chem. 264:17615-17618 (1989),
which is incorporated herein by reference), allowing the
production of soluble recombinant enzymes for large-scale
preparative synthesis (Colley et al., J. Biol.

10 Chem. 264:17619-17622 (1989)).

     To obtain sufficient amounts of fucosyltransferase
for large-scale reaction, the enzyme can be cloned and
expressed as a recombinant soluble enzyme by someone with
ordinary skill in the art. As a preferred method RNA can be

15 subtracted from the wild type CHO cells and LEC11 cells as
described by Chirgwin et al., Biochemistry 18:5214-5299 (1979),
and the poly A+ RNA isolated by chromatography on oligo(dT)-
cellulose. Next, cDNA from the LEC-11 cells can be prepared as
described by Sambrook et al., Molecular Cloning: A Laboratory

20 Manual, 2nd Ed. (1989), Cold Spring Harbor Press, New York,
which is incorporated herein by reference. The cDNA can be
subtracted using the method of Davis (Handbook of Experimental
Immunology, Vol. 2, pp. 1-13 (1986)) using excess poly A+ RNA
from wild type CHO cells, which do not express the desired

25 fucosyltransferase, but otherwise have most of the mRNA species
of LEC11 cells. A cDNA library can then be constructed in the
CDM8 expression vector using the subtracted cDNA (Seed,
Nature 329:840-842 (1987)). Clones expressing the
fucosyltransferase can be isolated using the expression cloning

30 method described by Larsen et al., Proc. Natl. Acad.
Sci. 86:8227-8231 (1989), employing transfection of COS-1 cells
and screening for cells expressing the SLX antigen with the
·CSLEX antibody or other antibody with specificity for the SLX
antigen. The full-length clone of the fucosyltransferase can

35 then be used to produce a soluble recombinant enzyme as taught
by Colley et al., supra.

     Another source of SLX is α_1-acid glycoprotein, which
is a plasma glycoprotein, the carbhydrate moieties of which can

be fucosylated to produce SLX (see, Alpha.-Acid glycoprotein: Genetics, Biochemistry, Physiological Functions, and Pharmacology, Bauman et al. ed. (Wiley 1989), and Walz, et al. Science 250:1132-1135 (1990), both of which are incorporated

5 herein by reference).

Although enzymatic or combined chemical and enzymatic synthesis of SLX compounds are preferred, chemical synthesis is also possible, as shown in Schemes II and IIa below.

Scheme II

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SLX-R

Scheme IIa

35

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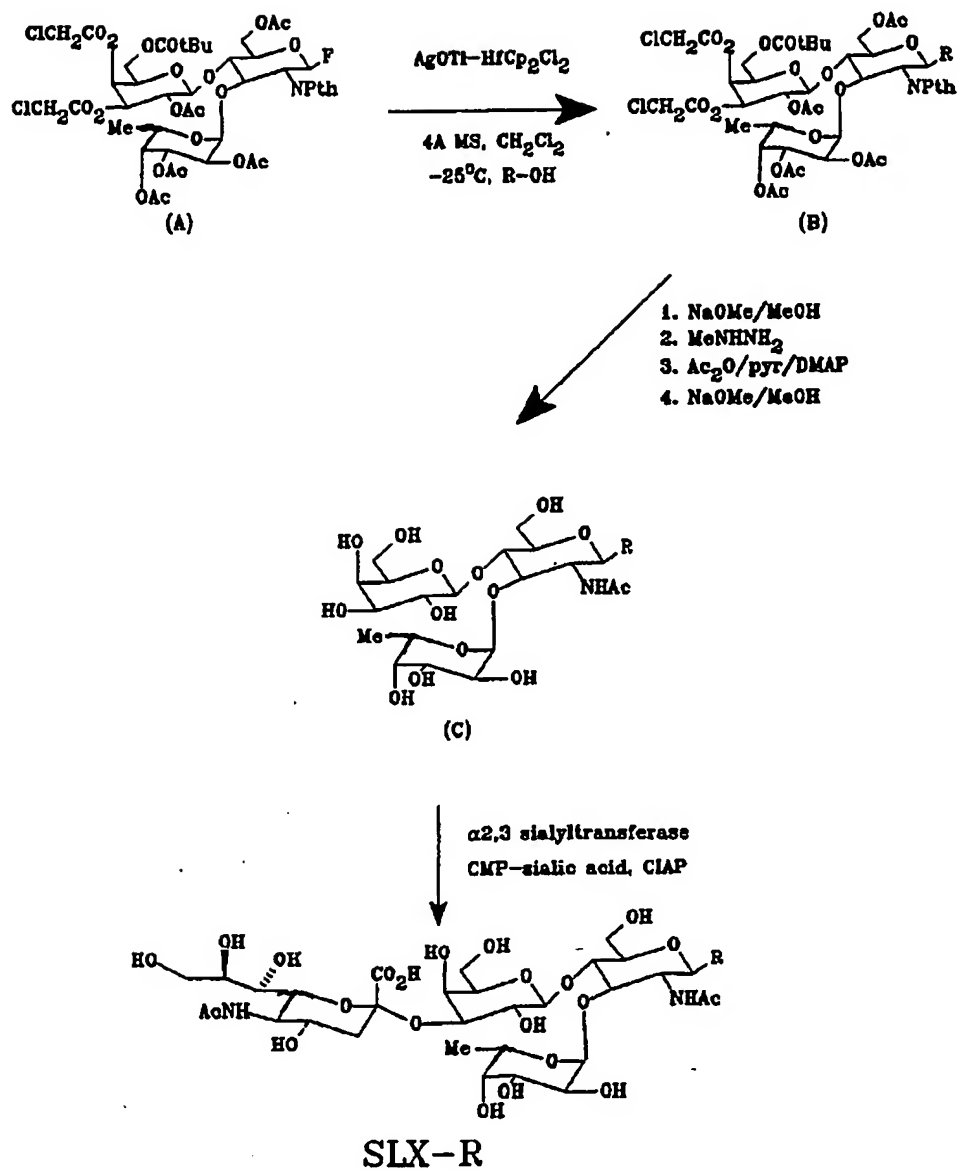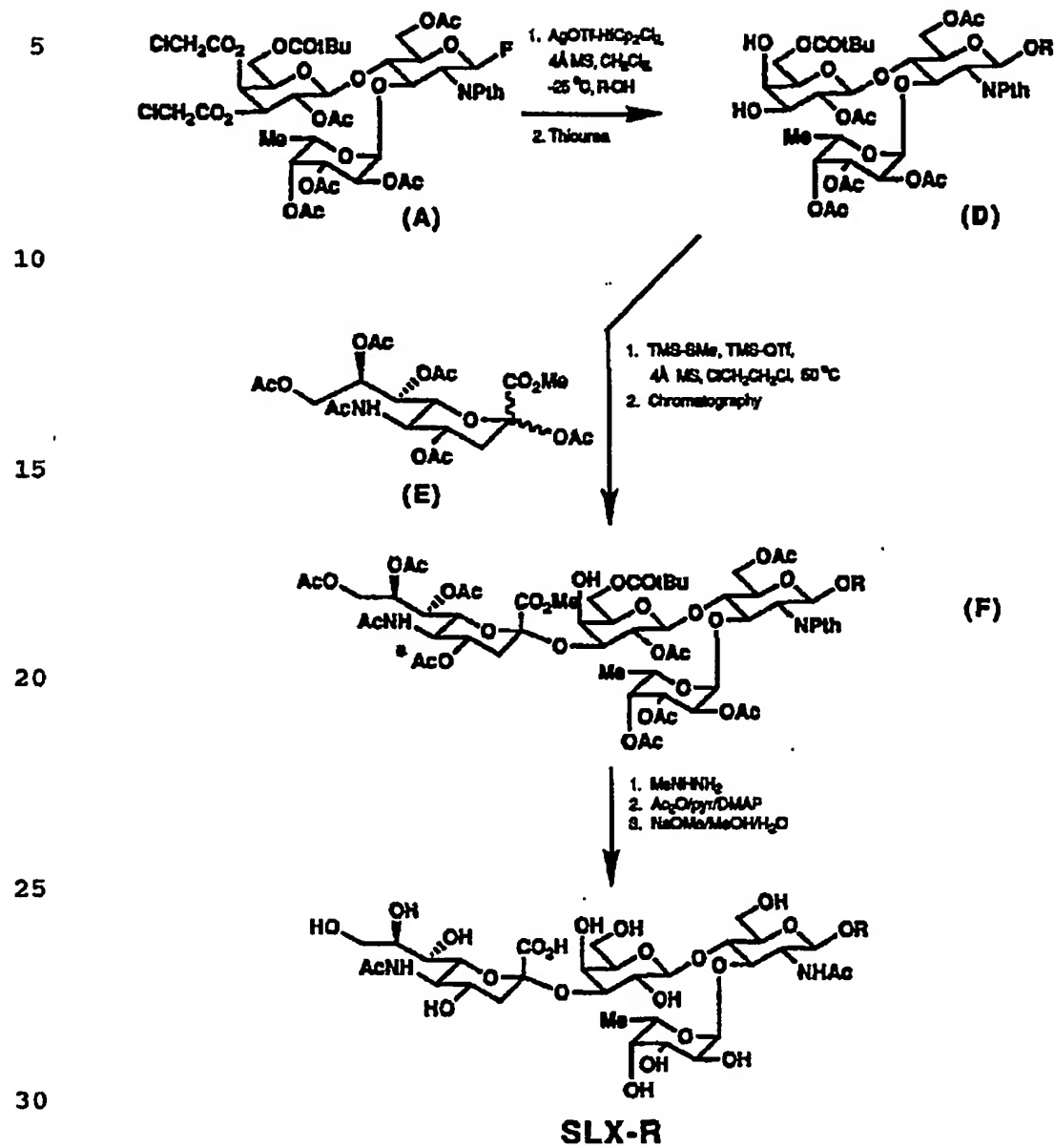
Component pieces of the SLX structure have been
synthesized. For instance, the preparation of sialic acid
containing glycosides, including SLX, is disclosed in European
Patent Application No. 88311312.8, which is incorporated herein
5   by reference. Nicolaou, et al., (J. Amer. Chem. Soc. 112:3693
(1990)) have published the total synthesis of the tumor-
associated Le[x] family of glycosphingolipids. Therein is
described the synthesis of the protected trisaccharide
Galβ1,4(Fucα1,3)GlcNAc (A) as illustrated in Scheme II.
10 Reaction of this intermediate with an appropriate glycosyl
acceptor (e.g., an alcohol moiety) results in compound (B).
Selective deprotection and acetylation of the glucosamine
moiety are carried out essentially as described in Nicolaou, et
al. to afford compound (C). Reaction of (C) with a
15 sialyltransferase as described above furnishes the desired
product SLX-R, although this may be produced in relatively low
yield using Scheme II.

Modified fucosides may be included in the synthetic
schemes to provide for SLX analogues which vary in this moiety.
20 For example, α-D-arabinosyl glycosides may be synthesized
following known procedures, Nicolaou et al., J. Amer. Chem. Soc.
112:3693-3695 (1990) through the use of tri-O-benzyl arabinosyl
halides. Other C-5 aryl or alkyl substituted arabinosyl
moieties may be synthesized, Danishefsky et al.,
25 J. Amer. Chem. Soc. 107:1274 (1985), Danishefsky, Aldrichimica
Acta. 19:59-68 (1986) and references therein, and introduced
into the disaccharide in the same manner. All of these
references are incorporated herein by reference.

According to alternative Scheme IIa, the
30 trisaccharide (A) is partially deprotected to furnish (D),
which is subsequently reacted with the peracetylated sialic
acid methyl ester (E) following a procedure described by
Kameyama et al., XV Intl. Carbohyd. Symp., Abst. No. A096,
(1990), and Carbihydrate Res., 209:c1-c4 (1991) (which are
35 incorporated herein by reference), yielding (F) after
chromatographic purification. Treatment of (F) sequentially
with methylhydrazine, N-acetylation, O-deacetylation and ester
hydrolysis furnishes SLX-R.

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Preferred examples of R for scheme II and IIa include
alkyl (straight chain, branched, saturated, mono- and poly-
unsaturated); serine (D or L); serine containing peptides; di-
and tri-alkanolamines (e.g. $[HO(CH_2)_n]_2NH$, $[HO(CH_2)_n]_3N$;
5   wherein $n = C_2-C_{20}$ as straight chain, branched, unsaturated,
mono- and poly-unsaturated). R can also be aryl, substituted
aryl (e.g., Me, OH, I; alone or in combination including ^{125}I),
alkylaryl, arylalkyl or other moiety, as the skilled artisan
would include for the desired use. The introduction of iodine
10   into phenolic compounds such as tyrosine is known in the art.
Radical groups containing phenols are useful for the
introduction of ^{125}I radioisotope, yielding compounds which are
useful in diagnosis.

Compounds comprising SLX and related structures may
15   also be used to assay for the presence of other compounds which
are capable of inhibiting intercellular adhesion mediated by
selectins. A number of methods can be used to assay the
biological activity of test compounds for the ability to
inhibit the selectin-mediated response. Ideally, the assays of
20   the present invention allow large scale _in vitro_ or _in vivo_
screening of a variety of compounds.

The agent or test compound to be screened will
typically be a synthetic or naturally-produced biomolecule,
such as a peptide, polypeptide, protein (_e.g._, monoclonal
25   antibody), carbohydrate (_e.g._, oligosaccharide),
glycoconjugate, nucleic acid, and the like. The compounds are
synthetically produced using, for instance, the methods for
synthesizing oligosaccharides described above (_see_, _also_,
Khadem, _Carbohydrate Chemistry_ (Academic Press, San Diego,CA,
30   1988), which is incorporated herein by reference). Methods for
synthesizing polypeptides of defined composition are well known
in the art (_see_, Atherton et al. _Solid Phase Peptide Synthesis_
(IRL Press, Oxford, 1989) which is incorporated herein by
reference). If the synthetic test compounds are polymeric
35   (_e.g._, polypeptides or polysaccharides) they are preferably
altered in a systematic way to identify the sequence of
monomers which have the desired effect (_see_, _e.g._, U.S. Patent
No. 4,833,092, which is incorporated herein by reference).

27

Test compounds may also be isolated from any natural source, such as animal, plant, fungal, or bacterial cells in accordance with standard procedures as described above. Potentially useful monoclonal antibodies can be prepared according to
5   standard methods described in more detail, below.

The assays of the present invention are particularly useful in identifying compounds which act as antagonists or agonists of a ligand molecule. Antagonists are compounds which reverse the physiological effect of a ligand or exclude binding
10   of the ligand to the receptor. An antagonist usually competes directly or indirectly with the ligand for the receptor binding site and, thus, reduces the proportion of ligand molecules bound to the receptor. Typically, an antagonist will be the topographical equivalent of the natural ligand and will compete
15   directly with the ligand for the binding site on the selectin. Such a compound is referred to here as a "mimetic." An SLX mimetic is a molecule that conformationally and functionally serves as substitute for an SLX moiety in that it is recognized by a selectin receptor. Alternatively, if the ligand and the
20   test compound can bind the receptor simultaneously, the compound may act non-competitively. A non-competitive inhibitor acts by decreasing or inhibiting the subsequent physiological effects of receptor-ligand interactions rather than by diminishing the proportion of ligand molecules bound to
25   the receptor. Finally, the assays of the present invention can be used to identify synthetic or naturally occurring agonists, that is, compounds which bind the receptor and initiate a physiological response similar to that of the natural ligand.

Numerous direct and indirect methods for in vitro
30   screening of inhibitors of ligand-receptor interactions are available and known to those skilled in the art. For instance, the ability to inhibit adhesion of SLX-bearing cells to cells expressing a particular selectin can be determined. As discussed above, selectin receptor genes have been cloned, thus
35   the genes can be inserted and expressed in a wide variety of cells, such as COS cells, CHO cells and the like. In addition, cells which do not normally express SLX are capable of being transformed with one or more glycosyltransferase genes which

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confer on the transformed cells the ability to synthesize the
ligand. (see, e.g., Lowe et al., Cell 63:475-484 (1990), which
is incorporated herein by reference.) Typically, the test
compound or agent is incubated with labelled SLX-bearing cells
5   and activated endothelial cells immobilized on a solid surface.
Inhibition of cellular adhesion is then determined by detecting
label bound to the surface after appropriate washes. In an
exemplified assay described below, promyelocytic HL-60 cells
and activated human endothelial cells or activated platelets
10 are used.

Since a ligand specific for selectin receptors has
now been identified, isolated ligand molecules can also be used
in the assays. The terms "isolated selectin-binding agent" or
"isolated SLX moiety" as used herein refer to a selectin
15 binding compound that is in other than its native state, e.g.,
not associated with the cell membrane of a cell that normally
expresses the ligand. Thus, an isolated SLX moiety may be a
component of an isolated molecule, such as an oligosaccharide
or a glycoconjugate. The isolated molecule may be synthesized
20 or prepared from the membranes of SLX-bearing cells.
Alternatively, the isolated selectin-binding agent or SLX
moiety may be associated with a liposome or attached to a solid
surface before use in the assay. Methods for preparing
selectin-binding liposomes and for immobilizing various
25 biomolecules are extensively discussed below.

Typically, the in vitro assays of the present
invention are competition assays which detect the ability of a
test compound to competitively inhibit binding of a compound
known to bind either the receptor or the ligand. Inhibition of
30 binding between SLX and a selectin receptor is usually tested.
Inhibition of other binding interactions are also suitable, for
instance, inhibition of the binding between a monoclonal
antibody (e.g., CSLEX-1) and SLX or between an SLX mimetic and
a selectin inhibitor can be used. Numerous types of
35 competitive assays are known (see, e.g., U.S. Patents No.
3,376,110, 4,016,043, and Harlow and Lane, Antibodies: A
Laboratory Manual, Cold Spring Harbor Publications, N.Y.
(1988), which are incorporated herein by reference).

29

The assays of the present invention are also suitable
for measuring binding of a test compound to one component alone
rather than using a competition assay. For instance,
immunoglobulins can be used to identify compounds that contain
5   the SLX moiety. Standard procedures for monoclonal antibody
assays, such as ELISA, may be used (see, Harlow and Lane,
supra). When assaying for glycolipids comprising the SLX
antigen, the reactivity of the monoclonal antibody with the
antigen can be assayed by TLC immunostaining by the method
10   originally described in Magnani et al., Anal.
Biochem. 109:399-402 (1980) or by solid-phase radioimmunoassay
as described by Kanagi et al., Cancer Res. 43:4997-5005 (1983);
which are incorporated herein by reference. Glycoproteins can
be assayed by standard immunoblotting procedures as described
15   in Harlow and Lane, supra. Sandwich assay formats are also
suitable. (see, e.g., U.S. Patent Nos. 4,642,285; 4,299,916; and
4,391,904; and Harlow and Lane, supra all of which are
incorporated herein by reference). Typically, compounds which
have been identified in a binding assay will be further tested
20   to determine their ability to inhibit receptor-ligand
interactions.

Other assay formats involve the detection of the
presence or absence of various physiological changes in either
ligand-bearing or selectin-bearing cells that result from the
25   interaction. Examples of suitable assays include the
measurement of changes in transcription activity induced by
binding (see, e.g., EPO Publication No. 3712820), the detection
of various cell mediated extra-cellular effects (see, e.g., PCT
Publication No. 90/00503), and the detection of changes in the
30   membrane potential of individual cells (see, e.g., U.S. Patent
No. 4,343,782), all of which are incorporated herein by
reference. Alternatively, conformational changes in isolated
·receptors or ligands can be detected; see, e.g., U.S. Patent
No. 4,859,609, which is incorporated herein by reference.
35   Any component of the assay, including the ligand, the
receptor, or the test compound, may be bound to a solid
surface. Many methods for immobilizing biomolecules on solid
surfaces are known in the art. For instance, the solid surface

may be a membrane (e.g., nitrocellulose), a microtiter dish
(e.g., PVC or polystyrene) or a bead. The desired component
may be covalently bound or noncovalently attached through
unspecific bonding.

5   A wide variety of organic and inorganic polymers,
both natural and synthetic may be employed as the material for
the solid surface.   Illustrative polymers include polyethylene,
polypropylene, poly(4-methylbutene), polystyrene,
polymethacrylate, poly(ethylene terephthalate), rayon, nylon,
10   poly(vinyl butyrate), silicones, polyformaldehyde, cellulose,
cellulose acetate, nitrocellulose, etc. Other materials which
may be employed, include paper, glasses, ceramics, metals,
metalloids, semiconductive materials, cermets or the like.   In
addition are included substances that form gels, such as
15   proteins, e.g., gelatins, lipopolysaccharides, silicates,
agarose and polyacrylamides or polymers which form several
aqueous phases, such as dextrans, polyalkylene glycols
(alkylene of 2 to 3 carbon atoms) or surfactants e.g.
amphiphilic compounds, such as phospholipids, long chain (12-24
20   carbon atoms) alkyl ammonium salts and the like. Where the
solid surface is porous, various pore sizes may be employed
depending upon the nature of the system.

              In preparing the surface, a plurality of different
materials may be employed, particularly as laminates, to obtain
25   various properties. For example, protein coatings, such as
gelatin can be employed to avoid non-specific binding, simplify
covalent conjugation, enhance signal detection or the like.

              If covalent bonding between a compound and the
surface is desired, the surface will usually be polyfunctional
30   or be capable of being polyfunctionalized. Functional groups
which may be present on the surface and used for linking can
include carboxylic acids, aldehydes, amino groups, cyano
groups, ethylenic groups, hydroxyl groups, mercapto groups and
the like. The manner of linking a wide variety of compounds to
35   various surfaces is well known and is amply illustrated in the
literature. See for example Immobilized Enzymes, Ichiro
Chibata, Halsted Press, New York, 1978, and Cuatrecasas, J.

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Biol. Chem. 245 3059 (1970) which is incorporated herein by
reference.

In addition to covalent bonding, various methods for
noncovalently binding an assay component can be used.
5   Noncovalent binding is typically nonspecific absorption of a
compound to the surface. Typically, the surface is blocked
with a second compound to prevent nonspecific binding of
labelled assay components. Alternatively, the surface is
designed such that it nonspecifically binds one component but
10 does not significantly bind another. For example, a surface
bearing a lectin such as Concanavalin A will bind a
carbohydrate containing compound but not a labelled protein
that lacks glycosylation. Various solid surfaces for use in
noncovalent attachment of assay components are reviewed in U.S.
15 Patent Nos. 4,447,576 and 4,254,082, which are incorporated
herein by reference.

Many assay formats employ labelled assay components
such as SLX ligands, SLX mimetics, immunoglobulins, receptors,
or test compounds. The label may be coupled directly or
20 indirectly to the desired component of the assay according to
methods well known in the art. A wide variety of labels may be
used. The component may be labelled by any one of several
methods. The most common method of detection is the use of
autoradiography with ^3H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P labelled
25 compounds or the like. The choice of radioactive isotope
depends on research preferences due to ease of synthesis,
varying stability, and half lives of the selected isotopes.
Other non-radioactive labels include ligands which bind to
labelled antibodies, fluorophores, chemiluminescent agents,
30 enzymes, and antibodies which can serve as specific binding
pair members for a labelled ligand. The choice of label
depends on sensitivity required, ease of conjugation with the
·compound, stability requirements, and available
instrumentation.
35   Non-radioactive labels are often attached by indirect
means. Generally, a ligand molecule (e.g., biotin) is
covalently bound to the molecule. The ligand then binds to an
anti-ligand (e.g., streptavidin) molecule which is either

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inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. Ligands and anti-ligands may be varied widely. Where a ligand has a natural anti-ligand, for

5   example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labelled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to

10 signal generating compounds, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives,

15 rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol. For a review of various signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by

20 reference.

As discussed above, in addition to various inhibitor compounds which comprise an accessible SLX unit or SLX mimetic, the present invention also provides monoclonal antibodies capable of inhibiting intercellular adhesion mediated by

25 selectins as well as methods for identifying such antibodies. The monoclonal antibodies bind a selectin ligand or receptor and block cellular adhesion. Thus, the multitude of techniques available to those skilled in the art for production and manipulation of various immunoglobulin molecules can be applied

30 to inhibit intercellular adhesion.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon

35 and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The immunoglobulins may exist in a variety of forms besides antibodies, including for example, Fv, Fab, and F(ab)$_2$, as well as in single chains

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(e.g., Huston et al., Proc. Nat. Acad. Sci. U.S.A. 85:5879-5883
(1988) and Bird et al., Science 242:423-426 (1988), which are
incorporated herein by reference). (see, generally, Hood et
al., Immunology, 2nd ed., Benjamin, N.Y. (1984), and

5   Hunkapiller and Hood, Nature 323:15-16 (1986), which are
incorporated herein by reference.)

            Antibodies which bind the SLX antigen may be produced
by a variety of means. The production of non-human monoclonal
antibodies, e.g., murine, lagomorpha, equine, etc., is well

10   known and may be accomplished by, for example, immunizing the
animal with the SLX antigen or a preparation containing a
glycoprotein or glycolipid comprising the antigen. Antibody-
producing cells obtained from the immunized animals are
immortalized and screened, or screened first for the production

15   of antibody which inhibits the interaction of the viral surface
protein with the receptor molecule and then immortalized. For
a discussion of general procedures of monoclonal antibody
production see Harlow and Lane, Antibodies, A Laboratory Manual
(1988), supra.

20   The generation of human monoclonal antibodies to a
human antigen (in the case of an SLX unit isolated from human
tissue) may be difficult with conventional techniques. Thus,
it may be desirable to transfer the antigen binding regions of
the non-human antibodies, e.g., the F(ab')$_2$ or hypervariable

25   regions, to human constant regions (Fc) or framework regions by
recombinant DNA techniques to produce substantially human
molecules. Such methods are generally known in the art and are
described in, for example, U.S. 4,816,397, EP
publications 173,494 and 239,400, which are incorporated herein

30   by reference. Alternatively, one may isolate DNA sequences
which encode a human monoclonal antibody or portions thereof
that specifically bind to the human SLX by screening a DNA
library from human B cells according to the general protocol
outlined by Huse et al., Science 246:1275-1281 (1989),

35   incorporated herein by reference, and then cloning and
amplifying the sequences which encode the antibody (or binding
fragment) of the desired specificity.

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A number of currently available monoclonal antibodies can be used according to the present invention to inhibit intercellular adhesion mediated by selectins. For instance, CSLEX-1 (see, Campbell et al., J. Biol. Chem. 259:11208-11214
5 (1984)}, VIM-2, which recognizes a sequence slightly different from SLX (see, Macher et al., supra), FH6 (described in U.S. Patent No. 4,904,596) (all references are incorporated herein by reference) or SH_3 and SH_4 generated by Dr. S. Hakomori of the Biomembrane Institute in Seattle, Washington.
10   The compounds of the present invention, including immunoglobulins, can be used in preparing pharmaceutical formulations as discussed below. If the compound is an oligosaccharide or glycoconjugate, the SLX or SLX-mimetic moiety can be presented in a variety of forms, but should be
15 able to effectively bind to a selectin receptor, such as ELAM-1, GMP-140, or MEL-14 antigen and thereby inhibit intercellular adhesion.

The pharmaceutical compositions of the present invention can be used to block or inhibit cellular adhesion
20 associated with a number of disorders. For instance, a number of inflammatory disorders are associated with selectins expressed on vascular endothelial cells and platelets. The term "inflammation" is used here to refer to reactions of both the specific and non-specific defense systems. A specific
25 defense system reaction is a specific immune system reaction to an antigen. Example of specific defense system reactions include antibody response to antigens, such as viruses, and delayed-type hypersensitivity. A non-specific defense system reaction is an inflammatory response mediated by leukocytes
30 generally incapable of immunological memory. Such cells include macrophages, eosinophils and neutrophils. Examples of non-specific reactions include the immediate swelling after a bee sting, and the collection of PMN leukocytes at sites of bacterial infection (e.g., pulmonary infiltrates in bacterial
35 pneumonias and pus formation in abscesses).

Other treatable disorders include, e.g., rheumatoid arthritis, post-ischemic leukocyte-mediated tissue damage (reperfusion injury), frost-bite injury or shock, acute

leukocyte-mediated lung injury (e.g., adult respiratory
distress syndrome), asthma, traumatic shock, septic shock,
nephritis, and acute and chronic inflammation, including atopic
dermatitis, psoriasis, and inflammatory bowel disease. Various
5   platelet-mediated pathologies such as atherosclerosis and
clotting can also be treated. In addition, tumor metastasis
can be inhibited or prevented by inhibiting the adhesion of
circulating cancer cells. Examples include carcinoma of the
colon and melanoma.
10   By way of example, reperfusion injury is particularly
amenable to treatment by compositions of the present invention.
Compositions which inhibit a GMP-140 selectin-ligand
interaction may be particularly useful for treating or
preventing reperfusion injury. The present invention may be
15   used prophylactically prior to heart surgery to enhance post-
surgical recovery.
            Because GMP-140 is stored in Weibel-Palade bodies of
platelets and endothelial cells and is released upon activation
by thrombin to mediate adhesion of neutrophils and monocytes,
20   inhibitors of the GMP-140 -ligand interaction may be especially
useful in minimizing tissue damage which often accompanies
thrombotic disorders. For instance, such inhibitors may be of
therapeutic value in patients who have recently experienced
stroke, myocardial infarctions, deep vein thrombosis, pulmonary
25   embolism, etc. The compounds are especially useful in pre-
thrombolytic therapy.
            Compositions of. the invention find particular use in
treating the secondary effects of septic shock or disseminated
intravascular coagulation (DIC). Leukocyte emigration into
30   tissues during septic shock or DIC often results in
pathological tissue destruction. Furthermore,.these patients
may have widespread microcirculatory thrombi and diffuse
·inflammation. The therapeutic compositions provided herein
inhibit leukocyte emigration at these sites and mitigates
35   tissue damage.
            The inhibitors of selectin-ligand interaction also
are useful in treating traumatic shock and acute tissue injury
associated therewith. Because the selectins play a role in

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recruitment of leukocytes to the sites of injury, particularly
ELAM-1 in cases of acute injury and inflammation, inhibitors
thereof may be administered locally or systemically to control
tissue damage associated with such injuries. Moreover,
5   because of the specificity of such inhibitors for sites of
inflammation, e.g., where ELAM-1 receptors are expressed, these
compositions will be more effective and less likely to cause
complications when compared to traditional anti-inflammatory
agents.
10   Thus, the present invention also provides
pharmaceutical compositions which can be used in treating the
aforementioned conditions. The pharmaceutical compositions are
comprised of biomolecules or other compounds which comprise an
SLX unit, antibodies which bind to SLX, or other compounds
15   which inhibit the interaction between the SLX ligand and
selectin receptors, together with pharmaceutically effective
carriers. A biomolecule of the present invention may be a
peptide, polypeptide, protein (e.g., an immunoglobulin),
carbohydrate (e.g., oligosaccharide or polysaccharide),
20   glycoconjugate (e.g., glycolipid or glycoprotein), nucleic
acid, and the like. The pharmaceutical compositions are
suitable for use in a variety of drug delivery systems. For a
brief review of present methods for drug delivery, see, Langer,
Science 249:1527-1533 (1990), which is incorporated herein by
25   reference.
            In light of the complexity of the inflammatory
response in mammals, one of skill will readily recognize that
the pharmaceutical compositions of the present invention may
comprise SLX bearing compounds in admixture with other
30   compounds known to interfere with the function of other
cellular adhesion molecules. For instance, members of the
integrin family of adhesion molecules are thought to play a
·role in the extravasation of leukocytes at points of infection.
For a review of intercellular adhesion receptors, including
35   selectin receptors, and their role immune function, see
Springer, Nature 346:425-434 (1990), which is incorporated
herein by reference. In addition, successful treatment using
the pharmaceutical compositions of the present invention may

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also be determined by the state of development of the condition to be treated. Since different adhesion molecules may be up or down regulated in response to a variety of factors during the course of the disease or condition, one of skill will recognize
5   that different pharmaceutical compositions may be required for treatment of different inflammatory states.

In one embodiment, the SLX ligand of the pharmaceutical composition can be used to target conventional anti-inflammatory drugs or other agents to specific sites of
10 tissue injury. By using a selectin-binding moiety such as an SLX ligand or SLX mimetic to target a drug to a selectin receptor on, e.g., a vascular endothelial cell, such drugs can achieve higher concentrations at sites of injury. Side effects from the conventional anti-inflammatory chemotherapeutic agents
15 can be substantially alleviated by the lower dosages, the localization of the agent at the injury sites and/or the encapsulation of the agent prior to delivery.

The targeting component, i.e., the SLX ligand or an SLX mimetic which binds to a desired selectin, can be directly
20 or indirectly coupled to the chemotherapeutic agent. The coupling, which may be performed by means, generally known in the art, should not substantially inhibit the ability of the ligand to bind the receptor nor should it substantially reduce the activity of the chemotherapeutic agent. A variety of
25 chemotherapeutics can be coupled for targeting. For example, anti-inflammatory agents which may be coupled include SLX-bearing compounds of the present invention, immunomodulators, platelet activating factor (PAF) antagonists, cyclooxygenase inhibitors, lipoxygenase inhibitors, and leukotriene
30 antagonists. Some preferred moieties include cyclosporin A, indomethacin, naproxen, FK-506, mycophenolic acid, etc. Similarly, anti-oxidants, e.g., superoxide dismutase, are useful in treating reperfusion injury when targeted by a SLX ligand or mimetic. Likewise, anticancer agents can be targeted
35 by coupling the SLX ligand or mimetic to the chemotherapeutic agent. Examples of agents which may be coupled include daunomycin, doxorubicin, vinblastine, bleomycin, etc.

The selectin receptor targeting may also be
accomplished via amphipaths, or dual character molecules
(polar:nonpolar) which exist as aggregates in aqueous solution.
Amphipaths include nonpolar lipids, polar lipids, mono- and
5   diglycerides, sulfatides, lysolecithin, phospholipids, saponin,
bile acids and salts. These molecules can exist as emulsions
and foams, micelles, insoluble monolayers, liquid crystals,
phospholipid dispersions and lamellar layers. These are
generically referred to herein as liposomes. In these
10   preparations the drug to be delivered is incorporated as part
of a liposome in conjunction with a SLX ligand or mimetic which
binds to the selectin receptor. Thus, liposomes filled with a
desired chemotherapeutic agent can be directed to a site of
tissue injury by the selectin-SLX ligand interaction. When the
15   liposomes are brought into proximity of the affected cells,
they deliver the selected therapeutic compositions.

        The liposomes of the present invention are formed
from standard vesicle-forming lipids, which generally include
neutral and negatively charged phospholipids and a sterol, such
20   as cholesterol. The selection of lipids is generally guided by
consideration of, e.g., liposome size and stability of the
liposomes in the bloodstream.

        Typically, the major lipid component in the liposomes
is phosphatidylcholine. Phosphatidylcholines having a variety
25   of acyl chain groups of varying chain length and degree of
saturation are available or may be isolated or synthesized by
well-known techniques. In general, less saturated
phosphatidylcholines are more easily sized, particularly when
the liposomes must be sized below about 0.3 microns, for
30   purposes of filter sterilization. Methods used in sizing and
filter-sterilizing liposomes are discussed below. The acyl
chain composition of phospholipid may also affect the stability
of liposomes in the blood. One preferred phosphatidylcholine
is partially hydrogenated egg phosphatidylcholine.
35   Targeting of liposomes using a variety of targeting
agents (e.g., ligands, receptors and monoclonal antibodies) is
well known in the art. (see, e.g., U.S. Patent Nos. 4,957,773
and 4,603,044, both of which are incorporated herein by

reference). Glycoproteins and glycolipids of a variety of
molecular weights can be used as targeting agents. Typically,
glycoproteins having a molecular weight less than about 300,000
daltons, preferably between about 40,000 and about 250,000 are
5   used, more preferably between about 75,000 and about 150,000.
Glycolipids of molecular weight of less than about 10,000
daltons, preferably between about 600 and about 4,000 are used.
    Standard methods for coupling targeting agents to
liposomes can be used. These methods generally involve
10 incorporation into liposomes of lipid components, such as
phosphatidylethanolamine, which can be activated for attachment
of targeting agents, or derivatized lipophilic compounds, such
as lipid derivatized bleomycin.. Antibody targeted liposomes
can be constructed using, for instance, liposomes which
15 incorporate protein A (see, Renneisen, et al., J. Biol. Chem.,
265:16337-16342 (1990) and Leonetti et al., Proc. Natl. Acad.
Sci. (USA) 87:2448-2451 (1990), both of which are incorporated
herein by reference).
    Targeting mechanisms generally require that the
20 targeting agents be positioned on the surface of the liposome
in such a manner that the target agents are available for
interaction with the selectin receptor. The liposome is
typically fashioned in such a way that a connector portion is
first incorporated into the membrane at the time of forming the
25 membrane. The connector portion must have a lipophilic portion
which is firmly embedded and anchored in the membrane. It must
also have a hydrophilic portion which is chemically available
on the aqueous surface of the liposome. The hydrophilic
portion is selected so that it will be chemically suitable to
30 form a stable chemical bond with the targeting agent which is
added later. Therefore, the connector molecule must have both
a lipophilic anchor and a hydrophilic reactive group suitable
for reacting with the target agent and holding the target agent
in its correct position, extended out from the liposome's
35 surface. In some cases it is possible to attach the target
agent to the connector molecule directly, but in most instances
it is more suitable to use a third molecule to act as a
chemical bridge, thus linking the connector molecule which is

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in the membrane with the target agent which is extended, three
dimensionally, off of the vesicle surface.

Liposome charge is an important determinant in
liposome clearance from the blood, with negatively charged
5   liposomes being taken up more rapidly by the
reticuloendothelial system (Juliano, Biochem. Biophys. Res.
Commun. 63:651 (1975)) and thus having shorter half-lives in
the bloodstream. Liposomes with prolonged circulation half-
lives are typically desirable for therapeutic and diagnostic
10   uses. Liposomes which can be maintained from 8, 12, or up to
24 hours in the bloodstream provide sustained release of the
selectin-ligand inhibitors of the invention, or may facilitate
targeting of the inhibitors (which may be labelled to provide
for in vivo diagnostic imaging) to a desired site before being
15   removed by the reticuloendothelial system.

Typically, the liposomes are prepared with about 5-15
mole percent negatively charged phospholipids, such as
phosphatidylglycerol, phosphatidylserine or
phosphatidylinositol. Added negatively charged phospholipids,
20   such as phosphatidylglycerol, also serves to prevent
spontaneous liposome aggregating, and thus minimize the risk of
undersized liposomal aggregate formation. Membrane-rigidifying
agents, such as sphingomyelin or a saturated neutral
phospholipid, at a concentration of at least about 50 mole
25   percent, and 5-15 mole percent of monosialylganglioside, may
provide increased circulation of the liposome preparation in
the bloodstream, as generally described in U.S. Pat. No. 4,
837,028, incorporated herein by reference.

Additionally, the liposome suspension may include
30   lipid-protective agents which protect lipids and drug
components against free-radical and lipid-peroxidative damages
on storage. Lipophilic free-radical quenchers, such as
alphatocopherol and water-soluble iron-specific chelators, such
as ferrioxianine, are preferred.

35   A variety of methods are available for preparing
liposomes, as described in, e.g., Szoka et al., Ann. Rev.
Biophys. Bioeng. 9:467 (1980), U.S. Pat. Nos. 4, 235,871,
4,501,728 and 4,837,028, incorporated herein by reference. One

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method produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired,

5   the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with an aqueous solution of the targeted drug and the targeting component and

10   allowed to hydrate, typically over a 15-60 minute period with agitation. The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

15   The hydration medium contains the targeted drug at a concentration which is desired in the interior volume of the liposomes in the final liposome suspension. Typically the drug solution contains between 10-100 mg/ml in a buffered saline. The concentration of the targeting SLX molecule or mimetic

20   which binds a selectin is generally between about 0.1 - 20 mg/ml.

             Following liposome preparation, the liposomes may be sized to achieve a desired size range and relatively narrow distribution of liposome sizes. One preferred size range is

25   about 0.2-0.4 microns, which allows the liposome suspension to be sterilized by filtration through a conventional filter, typically a 0.22 micron filter. The filter sterilization method can be carried out on a high through-put basis if the liposomes have been sized down to about 0.2-0.4 microns.

30   Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles

35   less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a

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standard emulsion homogenizer until selected liposome sizes,
typically between about 0.1 and 0.5 microns, are observed. In
both methods, the particle size distribution can be monitored
by conventional laser-beam particle size discrimination.

5   Extrusion of liposome through a small-pore
polycarbonate membrane or an asymmetric ceramic membrane is
also an effective method for reducing liposome sizes to a
relatively well-defined size distribution. Typically, the
suspension is cycled through the membrane one or more times
10   until the desired liposome size distribution is achieved. The
liposomes may be extruded through successively smaller-pore
membranes, to achieve a gradual reduction in liposome size.
            Even under the most efficient encapsulation methods,
the initial sized liposome suspension may contain up to 50% or
15   more drug and targeting agent in free (non-encapsulated) form.
Therefore, to maximize the advantages of liposomal targeted
drug, it is important to remove free drug and targeting agent
from the final injectable suspension.
            Several methods are available for removing non-
20   entrapped compound from a liposome suspension. In one method,
the liposomes in the suspension are pelleted by high-speed
centrifugation leaving free compound and very small liposomes
in the supernatant. Another method involves concentrating the
suspension by ultrafiltration, then resuspending the
25   concentrated liposomes in a drug-free replacement medium.
Alternatively, gel filtration can be used to separate large
liposome particles from solute molecules.
            Following treatment to remove free drug and/or
targeting agent, the liposome suspension is brought to a
30   desired concentration for use in intravenous administration.
This may involve resuspending the liposomes in a suitable
volume of injection medium, where the liposomes have been
·concentrated, for example by centrifugation or ultrafiltration,
or concentrating the suspension, where the drug removal step
35   has increased total suspension volume. The suspension is then
sterilized by filtration as described above. The liposome-
ligand preparation may be administered parenterally or locally
in a dose which varies according to, e.g., the manner of

administration, the drug being delivered, the particular
disease being treated, etc.

     For pharmaceutical compositions which comprise the
SLX ligand, and/or SLX mimetics which bind to selectin
5   receptors, the dose of the compound will vary according to,
e.g., the particular compound, the manner of administration,
the particular disease being treated and its severity, the
overall health and condition of the patient, and the judgment
of the prescribing physician. For example, for the treatment
10 of reperfusion injury, the dose is in the range of about 50 μg
to 2,000 mg/day for a 70 kg patient. Ideally, therapeutic
administration should begin as soon as possible after the
myocardial infarction or other injury. The pharmaceutical
compositions are intended for parenteral, topical, oral or
15 local administration, such as by aerosol or transdermally, for
prophylactic and/or therapeutic treatment. The pharmaceutical
compositions can be administered in a variety of unit dosage
forms depending upon the method of administration. For
example, unit dosage forms suitable for oral administration
20 include powder, tablets, pills, capsules and dragees.

     Preferably, the pharmaceutical compositions are
administered intravenously. Thus, this invention provides
compositions for intravenous administration which comprise a
solution of the compound dissolved or suspended in a
25 pharmaceutically acceptable carrier, preferably an aqueous
carrier. A variety of aqueous carriers may be used, e.g.,
water, buffered water, 0.4% saline, and the like. These
compositions may be sterilized by conventional, well known
sterilization techniques, or may be sterile filtered. The
30 resulting aqueous solutions may be packaged for use as is, or
lyophilized, the lyophilized preparation being combined with a
sterile aqueous solution prior to administration. The
compositions may contain pharmaceutically acceptable auxiliary
substances as required to approximate physiological conditions,
35 such as pH adjusting and buffering agents, tonicity adjusting
agents, wetting agents and the like, for example, sodium
acetate, sodium lactate, sodium chloride, potassium chloride,

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calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

The concentration of SLX ligand or mimetic, which may be combined with other SLX ligands or mimetics to form a
5   "cocktail" for increased efficacy in the pharmaceutical formulation, can vary widely, i.e., from less than about 0.05%, usually at or at least about 1% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of
10 administration selected. The cocktail may also comprise a monoclonal antibody which binds to selectin receptor, e.g., a monoclonal antibody to ELAM-1 or GMP-140, combined with the SLX ligand, a ligand mimetic or a monoclonal antibody to the ligand, so as to effectively inhibit the ligand-receptor
15 interaction. As described above, the cocktail components may be delivered via liposome preparations.

Thus, a typical pharmaceutical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 25 mg of the compound. Actual
20 methods for preparing parenterally administrable compounds will be known or apparent to those skilled in the art and are described in more detail in for example, Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA (1985), which is incorporated herein by reference.

25   For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a
30 pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more SLX ligands or mimetics of the invention, preferably about 20% (see, Remington's,
35 supra).

For aerosol administration, the compounds are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of SLX

oligosaccharide ligands or mimetics are 0.05% - 30% by weight,
preferably 1% - 10%. The surfactant must, of course, be
nontoxic, and preferably soluble in the propellant.
Representative of such agents are the esters or partial esters

5   of fatty acids containing from 6 to 22 carbon atoms, such as
caproic, octanoic, lauric, palmitic, stearic, linoleic,
linolenic, olesteric and oleic acids with an aliphatic
polyhydric alcohol or its cyclic anhydride such as, for
example, ethylene glycol, glycerol, erythritol, arabitol,

10 mannitol, sorbitol, the hexitol anhydrides derived from
sorbitol, and the polyoxyethylene and polyoxypropylene
derivatives of these esters. Mixed esters, such as mixed or
natural glycerides may be employed. The surfactant may
constitute 0.1%-20% by weight of the composition, preferably

15 0.25-5%. The balance of the composition is ordinarily
propellant. Liquefied propellants are typically gases at
ambient conditions, and are condensed under pressure. Among
suitable liquefied propellants are the lower alkanes containing
up to 5 carbons, such as butane and propane; and preferably

20 fluorinated or fluorochlorinated alkanes. Mixtures of the
above may also be employed. In producing the aerosol, a
container equipped with a suitable valve is filled with the
appropriate propellant, containing the finely divided compounds
and surfactant. The ingredients are thus maintained at an

25 elevated pressure until released by action of the valve.
        The compositions containing the compounds can be
administered for prophylactic and/or therapeutic treatments.
In therapeutic applications, compositions are administered to a
patient already suffering from a disease, as described above,

30 in an amount sufficient to cure or at least partially arrest
the symptoms of the disease and its complications. An amount
adequate to accomplish this is defined as "therapeutically
effective dose." Amounts effective for this use will depend on
the severity of the disease and the weight and general state of

35 the patient, but generally range from about 0.5 mg to about
2,000 mg of SLX oligosaccharide or SLX mimetic per day for a 70
kg patient, with dosages of from about 5 mg to about 200 mg of
the compounds per day being more commonly used.

In prophylactic applications, compositions containing the compounds of the invention are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a "prophylactically effective
5   dose." In this use, the precise amounts again depend on the patient's state of health and weight, but generally range from about 0.5 mg to about 1,000 mg per 70 kilogram patient, more commonly from about 5 mg to about 200 mg per 70 kg of body weight.
10   Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of SLX oligosaccharide or SLX mimetic of this invention sufficient to
15   effectively treat the patient.

The compounds may also find use as diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient suspected of having an inflammation. For this use, the
20   compounds can be labeled with ^{125}I, ^{14}C, or tritium.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLE I
25   Isolation of α1,3-fucosyltransferase I from Golgi Apparatus

LEC11, HL-60, HT-29, certain adenocarcinomas (colo 205 cells in particular), and polymorphonuclear leukocytes (PMN, neutrophils) contain a very specific α1,3-fucosyltransferase I, which is able to transfer fucose from
30   GDP-fucose to the sialylated substrates NeuAcα2,3Galβ1,4GlcNAc or NeuGcα2,3Galβ1,4GlcNAc.

It is well known in the art that fucose is transferred to oligosaccharide chains in the lumen of the Golgi apparatus via specific fucosyltransferases, reviewed in
35   Schacter and Roseman, in "The Biochemistry of Glycoproteins and Proteoglycans", W. Lennarz, ed., Plenum Press, New York, pp. 85-160 (1980), which is incorporated herein by reference. Since the subcellular localization of the fucosyltransferases

is in the Golgi apparatus, the first step in the isolation of these enzymes is to isolate a Golgi apparatus fraction from a cell line which expresses this novel and specific α1,3-fucosyltransferase.

5   Golgi apparatus-derived vesicle fractions are prepared by a modification of the procedure described by Balch et al., Cell, 39:405 (1984) which is incorporated herein by reference. The LEC11, HL-60, HT-29, PMN, colo 205 or other cell lines containing the α1,3-fucosyltransferase I are grown

10   in suspension to a density of approximately 5 X 10^5 cells/ml. Cells are harvested from the suspension culture by centrifugation at 2,000 X g. The resulting cell pellet from a 12 liter suspension (6 X 10^9 cells) is resuspended in 3 volumes (packed cell volume) of ice-cold 0.25M sucrose (w/v) solution

15   containing Tris-Cl (10mM), pH 7.0, heat inactivated fetal calf serum (7%), and Aprotinin (100 μg/ml, Sigma Chemical, Co. St. Louis, Mo.).

          The cells are disrupted (approximately 60 strokes) with a tight fitting Wheaton glass dounce homogenizer using the

20   A pestle. · The homogenate is centrifuged for 5 min. at 500 X g in a table-top clinical centrifuge. Lipid and insoluble material remaining at the top of the solution in the centrifuge tube is discarded. The cloudy supernatant is transferred to a clean tube, and the sucrose concentration of the supernatant

25   fraction is then adjusted to 40% (w/v) sucrose in Tris-Cl (20 mM), pH 7.0, with the aid of a refractometer. Five milliliters of this suspension is transferred to an ultracentrifuge tube and is layered sequentially with 2.5 ml of 35% (w/v) sucrose in Tris-Cl (10 mM, pH 7.0) and 2.0 ml of 29% (W/v) sucrose in 10

30   mM Tris-Cl buffer. The gradient is centrifuged for 1 hr. at 110,000 X g in a SW-41 rotor (Beckman) at 5°C. Golgi apparatus enriched vesicles are collected from the 29% to 35% sucrose ·interphase. Other subcellular fractions are found at other interphases in the gradient; e.g., vesicles derived from the

35   rough and smooth endoplasmic reticulum band below the Golgi derived vesicles, etc. The band removed from the 29% to 35% interphase is analyzed for the presence and amount of sialyltransferase activity.

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The enzyme sialytransferase is only known to be found within Golgi apparatus-derived vesicles and is used by those trained in the art as a marker to assess the authenticity of the band collected from the 29-35% interphase.

5   Sialyltransferase assays are performed using asialofetuin as the acceptor as described by Briles et al., *J. Biol. Chem.*, 252:1107 (1977). A good Golgi apparatus derived vesicle preparation from LEC cells typically has a sialyltransferase-specific activity of 3.0 nmole/mg protein/hr.

10   The resulting Golgi apparatus preparation is then used as a source of the $\alpha 1,3$-fucosyltransferase I used in the enzymatic synthesis described above.

EXAMPLE II

15   Demonstration of Intercellular Adhesion
by Cells Expressing SLX

The ability of LEC11 cells (which express SLX) to bind to activated endothelial cells expressing ELAM-1 was compared to that of CHO cells and another glycosylation mutant,

20   LEC12, which expresses the structure Le^X, a non-sialylated form of SLX.

MATERIALS

Passage 5 human umbilical vein endothelial cells

25   (HUVEC) (Clonetics) which had been grown on a gelatin coated 48 well assay plate were used as the source of endothelial cells. Cells were stimulated with IL-1β (Genzyme) at 30 μg/ml. Cells were stimulated for exactly 4 hrs. HL-60 cells provided by American Type Culture Collection (ATCC No. CCL 240) were used

30   as the source of control ligand bearing cells. These were harvested from bulk culture in RPMI 1640 (Gibco) containing Penicillin (100 units/ml)/ Streptomycin (100 Mcg/ml)(Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10% Fetal Bovine Serum (Hazleton) (hereafter referred to as CRPMI).

35   LEC11, LEC12 and CHO-K1 were provided by Dr. P. Stanley. They were grown in suspension culture in complete alpha MEM containing ribonucleotides and deoxyribonucleotides (Gibco), Penicillin (100 units/ml)/ Streptomycin (100 μg/ml)(Irvine

Scientific), L-Glutamine (2mM) (Irvine Scientific) and 10%
Fetal Bovine Serum (Hazelton).

PROCEDURE

5   1.   HL-60, LEC11, LEC12 and CHO-K1 cells were
harvested and washed in CRPMI. A viable cell count was made
using trypan blue. 3×10^6 cells of each type were pelleted in
a 10 ml test tube and 300 μl of ^{51}Cr (450 μCi) (New England
Nuclear) was added to each pellet. The tubes were allowed to
10 incubate 1 hour at 37°C with gentle agitation.

         2.   Labeled cells were washed 3X in medium and
resuspended to 2×10^5/ 400 μl (6ml). The tubes were then
placed in a 4°C ice bath.
15

         3.   After 4 hours incubation with IL-1β the assay
plate containing activated HUVEC was removed from the incubator
and chilled for 15 minutes by placing the plate in a· 4°C ice
bath.
20

         4.   When the temperature in both samples had
equilibrated, the medium was removed from the assay wells with
a pasteur pipette a few wells at a time.

25   5.   Labeled cells were added to the wells in 400 μl
volumes equal to 2×10^5 cells/well. Three 400 μl aliquots of
each cell suspension were placed in glass tubes for
determination of input CPMs.

30   6.   The plate was incubated in the ice water bath
for 30 minutes.

         7.   Unbound cells were removed from the wells of the
assay plate by systematic resuspension using a pasteur pipette
35 followed by addition and removal of 0.7 ml of medium.

         8.   All of the medium was removed from the wells and
a solution of 0.125 M Tris, 2% SDS and 10% glycerin was added

(0.3 ml). The plate was allowed to stand for 30 minutes and then 0.5 ml of dH_2O was added to each well.

        9.   The fluid in each well was resuspended with a
P1000 pipette and transferred to a glass test tube. The P1000 tip was ejected into the tube.

        10.   The tubes, including those containing the input CPM samples were counted in a gamma counter.

        11.   CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. The mean and standard deviation of triplicate assay points were plotted.

        The results obtained in this experiment, shown in Fig. 1, indicate that cells expressing SLX have the ability to bind effectively to activated vascular endothelial cells expressing ELAM-1. These data show that LEC11 cells which express high levels of the unique carbohydrate SLX bind exceptionally well to IL-1β activated HUVEC, while LEC12 and CHO-K1 which lack significant quantities of this carbohydrate are poor binders of the activated HUVEC. This conclusion is further supported by the observation that this binding occurs at 4°C, a characteristic of ELAM-1 mediated binding.

<u>EXAMPLE III</u>
<u>Inhibition of Intercellular Adhesion by</u>
<u>Monoclonal Antibodies Specific for SLX.</u>

        Two sets of experiments are described which confirm that the ligand on neutrophils for ELAM-1 contains an oligosaccharide where the terminal sugars are NeuAcα2,3Galβ1,4(Fuc α1,3)GlcNac(SLX).

        These experiments are performed by assaying the ability of monoclonal antibodies specific for sialylated Le[X] and for the unsialylated form, Le[X], to block the ELAM-1 mediated adhesion of HL-60 cells to IL-1β stimulated HUVEC.

A.   <u>Monoclonal Antibody Panel 1</u>

Materials: Passage 3 HUVEC from cultures initiated
for the present experiments were used as described above.   Two
sets of triplicate wells were left unstimulated as controls.
Four triplicates were stimulated with IL-1β (Genzyme) at 10
5   μg/ml and 4 at 20 μg/ml. Cells were stimulated for exactly 4
hours.   HL-60 cells obtained from the American Type Culture
Collection were used as the source of ligand bearing cells.
These were harvested from bulk culture in RPMI-1640 (Gibco)
containing penicillin (100 units/ml), streptomycin (100 μg/ml)
10   (Irvine Scientific), L-Glutamine (2mM) (Irvine Scientific) and
10% Fetal Bovine Serum (Hazleton) (hereafter referred to as
cRPMI).

Monoclonal antibody preparations included SNH3 (IgM)
at about 20 μg/ml and SH1 (IgG3) at about 10 μg/ml.   The
15   specificity of SNH3 is for SLX, while SH1 recognizes the
unsialylated structure.
Procedure:

1.   HL-60 cells were harvested and washed in CRPMI.
A viable cell count was made using trypan blue.   3 x 10^6 cells
20   were placed in each of 2, 10 ml test tubes and 300 μl of ^{51}Cr
(450 μCi) (New England Nuclear) was added to each tube.   The
tubes were allowed to incubate 1 hour at 37°C with gentle
agitation.

25   2.   The antibodies were supplied as hybridoma
culture supernatants and contained 0.01% NaN3 and 0.05%
thimerosal.   To remove these preservatives, 5 ml. of each
antibody was dialysed against 3 changes of 500 ml each of
outdated tissue culture medium over 72 hours.
30

3.   Antibodies were collected from dialysis and 3.5
ml of each was placed in 10 ml tubes.   The remainder was
-retained for use in an ELISA assay for HL-60 binding.   7 ml of
RPMI 1640 5% FCS was placed in a 4th tube for use as a control.
35

4.   Labeled HL-60 cells were washed 3X in CRPMI and
pooled into one tube.   They were then centrifuged and
resuspended to 1 ml in medium.

5.   200 μl of cell suspension was added to each of the antibody containing tubes and 400 μl to the control tube. Tubes were incubated 20 min. at 37°C with gentle agitation.

5   6.   The stimulated HUVEC assay plate was removed from the incubator and the medium was removed from the wells with a pasteur pipette, a few wells at a time.

7.   0.5 ml of cell suspension was added to each of
10   triplicate wells. Control cells were plated on unstimulated and stimulated HUVEC at both IL-1β concentrations. Test cells were added to stimulated wells only.

8.   0.5 ml aliquots of each cell suspension were
15   added to glass tubes to be used to determine the input CPMs.

9.   The assay plate was returned to the incubator (5% CO_2, 37°C) for 30 min.

20   10.   An aliquot of each cell suspension was mixed with an equal volume of trypan blue and the cells were examined microscopically for viability. The results were: Control = 98%, SH1 = 92%, and SNH3 = 99%.

25   11.   Unbound cells were removed from the wells of the assay plate by systematic resuspension using a pasteur pipette followed by addition and removal of 0.7 ml of medium.

12.   All of the medium was removed from the wells and
30   a solution of 0.125 M Tris, 2% SDS (Bio-Rad) and 10% glycerin (Fisher) was added (0.3 ml). The plates were allowed to stand for 30 min. and then 0.6 ml of dH_2O was added to each well.

13.   The fluid in each well was resuspended with a
35   P1000 pipette and transferred to a glass test tube. The P1000 tip was ejected into the tube.

14. The tubes, including those containing the input counts per minute (CPM) samples were counted in a gamma counter.

5   15. CPMs bound in each well were divided by the input CPMs for each sample to determine the % bound. The mean and standard deviation of triplicate assay points were plotted.

           Replicates were judged to be best in the experiment
10 in which high IL-1β was used to induce the endothelial cells.
           The results showed that the monoclonal antibody SNH3 blocked the binding of HL-60 cells to IL-1β stimulated HUVEC via the ELAM-1 receptor. The control antibody SH1 which does not bind the SLX determinant did not block binding of HL-60
15 cells to ELAM-1. This suggests that the terminal sialic acid in the ligand is necessary for binding to ELAM-1.

B. Monoclonal Antibody Panel 2
           Materials: Passage 3 HUVEC which had been grown on
20 gelatin coated 48 well assay plates (Costar) were used as the source of endothelial cells. The plates were prepared as previously described above. Two sets of triplicate wells were left unstimulated as controls. Seven triplicates on each plate were stimulated with IL-1β at 30 μg/ml in 0.5 ml of EGM-UV.
25 Cells were stimulated for exactly 4 hrs. HL-60 cells (ATCC) were used as the source of ligand bearing cells. These were harvested from bulk culture in CRPMI. Fresh hybridoma supernatants containing monoclonal antibodies included: FH6 (IgM) a lower affinity mAb; SNH-3 (IgM) (20 μg/ml); SH-1 (IgG$_3$)
30 (10 μg/ml); FH-2 (IgM) a Le^x reactive mAb; SNH-4 (IgG3) a high affinity antibody; and CSLEX-1 (IgM) (provided by Dr. P. Terasaki, UCLA as purified immunoglobulin at 2.8 mg/ml, diluted to 9 μg/ml in Dulbecco's Modified Eagles Medium (DMEM) containing 5% FCS for use in this assay). The specificities of
35 the antibodies were as follows: FH6, SNH-4, SNH3 and CSLEX-1 were specific for SLX; FH2 and SH1 were specific for the unsialylated Le^x.
           Procedure:

54

1.   HL-60 cells were harvested and washed in CRPMI.
A viable cell count was made using trypan blue. 3 x 10^6 cells
were placed in each of 2, 10 ml test tubes and 300 μl of ^{51}Cr
(450 μCi) (New England Nuclear) was added to each tube. The
5   tubes were allowed to incubate 1 hour at 37°C with gentle
agitation.

2.   Labeled HL-60 cells were washed 3X in DMEM
containing 5% FCS (hereafter referred to as cDMEM) and pooled
10   into one tube. They were then centrifuged and resuspended to 4
x 10^6 cells per ml in the same medium.

3.   3.2 ml of each monoclonal antibody culture
supernatant, and 3.2 ml purified CSLEX-1 (29 μg), were added to
15   separate test tubes; a control tube received 6.4 ml of medium.

4.   200 μl of cell suspension (equal to about 8 x
10^5 cells) was added to tubes containing the monoclonal
antibodies and 400 μl to the control tube. Tubes were then
20   incubated 20 min. at 37°C with gentle agitation.

5.   The stimulated HUVEC assay plate was removed
from the incubator and the wells were washed one time with
cDMEM and the medium was removed from the wells with a pasteur
25   pipette, a few wells at a time.

6.   0.4 ml of cell suspension was added to each well
of one of the two plates. Control cells were plated on
unstimulated and stimulated HUVEC. Antibody treated cells were
30   added to stimulated wells only.

7.   0.4 ml aliquots of each cell suspension were
·added to glass tubes to be used to determine the input CPMs.

35   8.   The assay plate was incubated at 37°C for 30
min.

9.   The remainder of each cell suspension and the assay plate were placed in an ice bath to chill for 20 min.

10.   The cell suspensions were plated on the chilled
5   plate as for the 37°C plate above. This plate was incubated for 30 min. at 40°C.

The remaining steps of the assay were performed as described in steps 11-15 of Section A above, except that in
10   step 12 the plates were allowed to stand for 15 min. rather than 30 min.

The results, shown in Fig. 2A, indicate that the monoclonal antibodies SNH-3, FH6, SNH-4 and CSLEX-1, all specific for SLX, significantly blocked the binding of HL-60
15   cells to IL-1β stimulated HUVEC via the ELAM-1 receptor when incubated at 37°C. The monoclonal antibodies specific for Le^X (FH2 and SH1) were not effective inhibitors. Thus, the ligand for ELAM-1 contains the sialylated Le^X antigen or a similar structure found in cell surface glycoproteins or glycolipids.
20   When incubated at 4°C (Fig. 2B), antibodies FH6 and SNH-3 (both IgM's) enhanced binding. In these tests there appeared to be significant agglutination of the HL-60 cells in the wells, which may account for this observation.

25   C.   Monoclonal Antibodies Block Adhesion of LEC11 Cells to
       Cells which Express ELAM-1
       In this set of experiments the ability of monoclonal antibodies specific for SLX and for the unsialylated form, Le^X, to block the ELAM-1 mediated adhesion of LEC11 cells (which
30   express SLX) and LEC12 cells (which express Le^X) to IL-1β stimulated HUVEC.

Materials: Passage 4 HUVEC served as the source of endothelial cells. The plates were prepared as previously described. Two sets of triplicate wells were left unstimulated
35   as controls. 7 triplicates on each plate were stimulated with IL-1β at 30 μg/ml in a 0.5 ml volume of EGM-UV. Cells were stimulated for exactly 4 hrs. LEC11 and LEC12 cells, described generally in Stanley et al., J. Biol. Chem., 263:11374 (1988),

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<u>supra</u>, were provided by Dr. P. Stanley. They were grown in
suspension culture in complete alpha MEM containing
ribonucleotides and deoxyribonucleotides (Gibco), penicillin
(100 unitslml)/streptomycin (100 µg/ml) (Irvine Scientific), L-
5 Glutamine (2mM) (Irvine Scientific) and 10% FBS (Hazelton).
The monoclonal antibodies used in these experiments are
described in Section B, above. They included: FH6, SNH-3, SH-
1, FH-2, SNH-4 and CSLEX-1.

10 Procedure:

    1.   LEC11 and LEC12 cells were harvested and washed
in CRPMI. A viable cell count was made using trypan blue. 3 x
10^6 cells of each cell line were placed in each of 2, 10 ml
test tubes and 300 µl of ^{51}Cr (450 µCi) (New England Nuclear)
15 was added to each tube. The tubes were allowed to incubate 1
hr. at 37°C with gentle agitation.

    2.   The radiolabeled cells were washed X3 in cDMEM
and pooled into one tube. They were then centrifuged and
20 resuspended to 4 x 10^6 cells per ml in the same medium.

    3.   1.6 ml of each monoclonal antibody supernatant,
and 1.6 ml purified CSLEX-1 (15 µg), were added to separate
test tubes; control tubes received 3.2 ml medium.
25

    4.   200 µl of cell suspension equal to 4 x 10^5 LEC11
or LEC12 cells were added to tubes containing the monoclonal
antibodies and 400 µl to the control tube. Tubes were
incubated 20 min. at 37°C with gentle agitation.
30

    5.   The stimulated HUVEC assay plate was removed
from the incubator and the wells were washed one time with
·cDMEM and the medium was removed from the wells with a pasteur
pipette, a few wells at a time.
35

    6.   The cell suspensions and the assay plate were
placed in an ice bath to chill for 20 min.

57

7.   0.4 ml of cell suspension was added to each well of the previously described assay plate. Control cells were plated on unstimulated and stimulated HUVEC. Antibody treated cells were added to stimulated wells only. Each assay was done in triplicate.

5

8.   0.4 ml aliquots·of each cell suspension were added to glass tubes to be used to determine the input CPMs.

10   9.   The assay plate was incubated for 30 min. at 4°C.

The remaining steps of the assay were performed as described in steps 11-15 of Section A, above, except that in step 12 the plates were allowed to stand for 15 min.

The results shown, in Figs. 3A and 3B, indicate that the monoclonal antibodies SNH-3, FH6, SNH-4 and CSLEX-1 (all specific for SLX) significantly blocked the binding of LEC11 cells to IL-1β stimulated HUVEC via the ELAM-1 receptor. LEC12 cells, which do not express the SLX epitope, did not bind the activated endothelium. The monoclonal antibodies specific for Le^X (FH2 and SH1) caused minor inhibition of LEC11 binding.

Further confirmation that SLX is a primary ligand for ELAM-1 receptor was provided by removing sialic acid from LEC 11 and HL-60 cells. In these experiments the treatment of LEC 11 and HL-60 cells prior to adhesion assays with Clostridium perfringens neuraminidase (sialidase), 1.6 U/ml (Type X, Sigma Chem. Co.) for 90 min. at 37°C during ^{51}Cr-labelling. The results, shown in Fig. 4, confirm that sialidase substantially reduced the adhesion of LEC 11 and HL-60 cells by 70-85% to activated endothelial cells.

EXAMPLE IV

Liposomes of Glycosphingolipids Block Binding of

SLX Cells to Activated Endothelial Cells

This Example describes the preparation of liposomes which contain various biosynthetically produced glycosphingolipids on which the terminal carbohydrate units are

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either SLX, Le^X, or similar but not identical compounds. The
ability of the liposomes which contain SLX or SLX mimetics to
block the binding of SLX-expressing HL-60 cells and LEC11 cells
to endothelial cells which have been stimulated to express
5   ELAM-1 by treatment with IL-1β is shown.

Materials: The glycosphingolipids used in this
experiment are shown in Table I; they were obtained from the
Biomembrane Institute, Seattle, WA, and were either purified or
biosynthetically produced and characterized by NMR and mass
10   spectrometry, as generally described in Hakomori, S. I., et
al., J. Biol. Chem., 259:4672 (1984), and Fukushi Y., et al.,
J. Biol. Chem. 259:10511 (1984), incorporated by referenced
herein. S-diLe^X (SLX) was synthesized enzymatically by adding
fucosyl residues using a colo 205 cell line as enzyme source
15   and SH as substrate. Nonsialylated diLe^X was similarly
synthesized using nLc6 as substrate and the cell line NCI H-69.
See Holmes et al., J. Biol. Chem. 260:7619 (1985), incorporated
by reference herein. SPG and SH were purified from bovine red
blood cells, and nLc6 was produced by chemical removal of the
20   terminal sialosyl residue from SH.

59

Table 1. Glycolipids tested for liposome inhibition of ELAM-1 mediated cell adhesion.

Generic	IUPAC	Structure
nLc_6	nLc_6	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
diLe[1]	III^{3}V^{3}Fuc$_2$nLc$_6$	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer $\qquad\qquad\quad$3$\qquad\qquad\qquad\qquad$3 $\qquad\qquad\quad\uparrow\qquad\qquad\qquad\qquad\uparrow$ $\qquad\qquad$Fucα1$\qquad\qquad\qquad$Fucα1
SPG	IV^{3}NeuAcnLc$_4$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
SH	VI^{3}NeuAcnLC$_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
s-diLe^x	III^{3}V^{3}Fuc$_2$VI^{3}NeuAcnLc$_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer $\qquad\qquad\qquad\quad$3$\qquad\qquad\qquad\qquad\qquad\qquad$3 $\qquad\qquad\qquad\quad\uparrow\qquad\qquad\qquad\qquad\qquad\qquad\uparrow$ $\qquad\qquad\quad$Fucα1$\qquad\qquad\qquad\qquad\qquad$Fuc$\alpha$1

PSIDOCS16700

60

Liposomes containing the glycosphingolipids were
formed as follows: 100 µg of glycolipid was added to 300 µg
phosphatidylcholine (Sigma, egg yolk) and 500 µg cholesterol
(Sigma) in chloroform-methanol (2:1) and the whole solution
5   evaporated to dryness by N_2 in 15 ml screwcap tubes.

Passage 3 HUVEC, which had been grown on to
confluence on a gelatin coated 48 well assay plate (Costar)
were used as the source of endothelial cells. The plates were
prepared as previously described. Two sets of triplicate wells
10   were left unstimulated as controls. 14 triplicates were
stimulated with IL-1β at 30 µg/ml in a 0.5 ml volume of EGM-UV.
Cells were stimulated for exactly 4 hrs. HL-60 cells and LEC11
cells were cultured as described above.

15   Procedure:

1.   One 48 well Costar cluster dish containing HUVEC
grown to confluence on gelatin was removed from the incubator
and the medium in each well was removed with a pasteur pipette
and replaced either with 0.5 ml fresh EGM-UV medium or with the
20   same medium containing 30 µg/ml IL-1β, and the plate then
returned to the incubator for 4 hrs.

2.   HL-60 cells and LEC11 cells were harvested and
washed in CRPMI. A viable cell count was made using trypan
25   blue. 6 x 10^6 cells of each cell type were radiolabeled as
follows: 3 x 10^6 cells of each type were placed in each of 2,
10 ml test tubes and 300 µl of ^{51}Cr (450 µCi) (New England
Nuclear) was added to each tube. The tubes were allowed to
incubate 1 hr. at 37°C with gentle agitation.
30

3.   Radiolabeled HL-60 and LEC11 cells were washed
3X in CRPMI and pooled into one tube. They were then
centrifuged and resuspended to 2 x 10^6 cells per ml in the same
medium.
35

4.   The stimulated HUVEC assay plate was removed
from the incubator and the wells were washed two times with
RPMI 1640 containing 5 mg/ml bovine serum albumin (BSA).

5.   Liposomes were prepared as follows: The
evaporated pellets were dissolved in 100 μl of absolute ethanol
and sonicated for 2 min. Two ml of PBS was added slowly to the
tubes over two minutes while continuing to sonicate. This
5   stock was diluted 1:10 in RPMI 1640 medium just prior to use
and 50 μl of a stock solution of BSA at 100 mg/ml was added to
each 1 ml of diluted liposomes to make a final concentration of
5 mg/ml BSA.

10   6.   The medium was removed from the wells of the
assay plate with a pasteur pipette, a few wells at a time, and
0.3 ml of liposome suspension was added to each of 6 IL-1β
stimulated assay wells. Control wells received the liposome
buffer containing ethanol, RPMI 1640 and BSA at the same
15   concentrations as in the liposome containing wells. Control
buffer was plated on unstimulated and stimulated HUVEC.
Liposome containing samples were added to stimulated wells
only.

20   7.   The plates were incubated for 40 min. at 37°C
and then 50 μl of ^{51}Cr labeled HL-60 or LEC11 cells were added
to the assay wells. Each cell line was assayed in triplicate
on each liposome preparation. The final concentration of cells
was 10^5/350 μl/well. Three aliquots of 50 μl of each cell
25   suspension were added to glass tubes to be used to determine
the input CPMs, and the assay plate incubated at 37°C for 30
min.

            8.   Unbound cells were removed from the wells of the
30   assay plates by systematic resuspension using a pasteur pipette
followed by addition and removal of 0.7 ml of medium. All of
the medium was removed from the wells and a solution of 0.125 M
Tris, 2% SDS and 10% glycerin was added (0.3 ml). The plates
were allowed to stand for 15 min. and then 0.5 ml of dH_2O was
35   added to each well.

            9.   The fluid in each well was resuspended and
transferred to a glass test tube. The pipette tip was ejected

into the tube. The tubes, including those containing the input
CPM samples, were counted in a gamma counter. CPMs bound in
each well were divided by the input CPMs for each sample to
determine the % bound. The means and standard error of
5   triplicate assay points were plotted.

As shown in Fig. 5, liposomes containing selected
glycolipids having terminal sequences which contained SLX (S-
diLe^X, Table 1) dramatically inhibited adhesion of HL-60 cells
10   to activated endothelial cells at 4°C. Liposomes containing
glycolipids with Le^X (di-Le^X) or other related carbohydrate
structures (Table 1) exhibited minimal inhibition that was not
dependent on the structure of the carbohydrate group. Similar
results were obtained with LEC 11 cell adhesion. When the
15   experiments were performed at 37°C, HL-60 cell adhesion was
reduced by liposomes containing glycolipids with the SLX
structure (S-diLe^X, 70%), and also to a lesser extent by
liposomes containing Le^X (di-Le^X, 40%) suggesting that Le^X may
also interact with ELAM-1, but with a lower affinity. These
20   experiments show that biosynthetically produced SLX or similar
SLX mimetic compounds when formulated into liposome
compositions can serve as therapeutic compounds for, e.g., the
reduction of leukocyte infiltration into inflammatory sites.

Jurkat cells bind to IL-1 activated endothelial cells
25   predominantly through the V-CAM (endothelial cell) – VLA-4
(Jurkat cell) adhesion pair (Wayner et al., J. Cell Biol.
109:1321), in contrast to the adhesion of HL-60 and LEC 11
cells to activated endothelial cells through the ELAM-1
receptor. Jurkat cell adhesion was not inhibited by liposomes
30   which contained SLX, but was completely inhibited by monoclonal
antibody to the α subunit of the integrin molecule VLA-4. This
result demonstrates that SLX liposome inhibition of HL-60 and
LEC 11 cells is not a stearic effect attributable to binding of
liposomes to endothelial cells, but supports the conclusion
35   that SLX liposomes inhibit the adhesion through a direct
competition with the ligand binding site of ELAM-1.

EXAMPLE V

Antibodies to SLX Inhibit GMP-140 Mediated
Binding on Activated Human Platelets

5   In this Example the ability of monoclonal antibodies
specific for SLX and for the unsialylated Le^x to block the GMP-
140 mediated adhesion of HL-60 cells to activated human
platelets was determined.

           Materials: HL-60 cells are described above and were
10   used as the source of ligand bearing cells. Jurkat cells were
used as the non-ligand bearing control. Monoclonal antibodies
SH-1, FH-2, SNH-4, and CSLEX-1 are also described above.

           Procedure:
15   1.   Blood was drawn from a normal human donor into a
syringe containing ACD anticoagulant (dextrose, 2.0 g; sodium
citrate 2.49 g; and citric acid 1.25 g; to 100 ml with dH_2O) at
a ratio of 6 parts blood to 1 part anticoagulant.

           Platelets were isolated by differential
20   centrifugation as follows: Blood was centrifuged at 800 rpm
(approx. 90 x g) for 15 min. at room temp. The supernatant was
collected and centrifuged at 1200 rpm (approximately 400 x g)
for 6 min. The supernatant was removed and centrifuged at 2000
rpm (1200 x g) for 10 min. to pellet the platelets. The
25   platelet button was washed 2 times with Tyrode-HEPES buffer, pH
6.5 (NaCl 8.0 g; KCl 0.2 g; $NaH_2PO_4 \cdot H_2O$ 0.057 g; $MgCl_2 \cdot 6H_2O$
0.184 g; $NaHCO_3$ 0.1 g; Dextrose, 1.0 g; and HEPES, 2.383 g;
bring to 1 L with DI water, adjust to pH 6.5 with 1N NaOH)
followed by one wash in PBS. Platelets were suspended to a
30   concentration of 10^8/ml in PBS.

           2.   Approximately 20 min. before the platelets were
finally resuspended, 48 well plates were coated with 0.1%
gelatin and incubated to 15 min. at 37°C. Excess gelatin was
35   removed by pipette immediately fore the addition of the
platelet suspension. Platelets were activated by the addition
of 0.25 units of thrombin/ml (Sigma T-6759) of platelet

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suspension. Platelets were allowed to stand at room
temperature for 20 min.

3.   To prepare bound, activated platelets, 300 μl of
5   the platelet suspension was added to each well of the gel
coated plate. The plate was incubated at 37°C to 15 min., then
spun at 800 rpm (90 xg) for 2 min. The unbound platelets were
removed by washing the plate 3 times with PBS.

10   4.   Since platelets possess highly reactive Fc
receptors, to prevent uptake of any aggregated IgG from the
antibody preparation, the platelet Fc receptors were blocked as
follows: Purified mouse IgG W6/32 (IgG_{2a}) at 27 mg/ml was
aggregated by heating at 63°C for 5 min. 300 μl of the heated
15   preparation at 20 μg/ml in PBS was added to each well of the
platelet-coated plate. The plate was incubated at 37°C for 15
min. then washed with PBS.

5.   HL-60 and Jurkat cells were harvested and washed
20   in CRPMI. A viable cell count was made using trypan blue, 3 x
10^6 cells of each type were placed in each of 2, 10 ml test
tubes and 300 μl of ^{51}Cr (450 μCi) (New England Nuclear) was
added to each tube. The tubes were incubated for 1 hr. at 37°C
with gentle agitation.
25

6.   Radiolabeled cells were washed 3X in CRPMI and
pooled into one tube. They were then centrifuged and
resuspended to 4 x 10^6 cells per ml in the same medium.

30   7.   1.6 ml of each monoclonal antibody culture
supernatant, and 1.6 ml of purified CSLEX-1 (15 μg) were added
to separate test tubes; control tubes received 1.6 ml of
· medium.

35   8.   100 μl of the labeled HL-60 of Jurkat cell
suspension (containing 4 x 10^5 cells) was added to each of the
tubes which contained monoclonal antibody. They were incubated
for 20 min. at 37°C with gentle agitation. Following this

65

incubation period, 0.3 ml of each cell suspension (containing
7.5×10^4 cells) was added to each well of the previously
described assay plate containing bound activated platelets.
Each assay was done in triplicate.

5

9.   The assay plate was centrifuged at 90 xg for 2
min. and then incubated for 5 min. at room temp.   Unbound cells
were removed from the wells of the assay plate by inverting the
plate into a radioactive waste receptacle and blotting the
10   plate on towels.   The wells were washed X3 by carefully adding
300 μl PBS to each well and inverting and blotting the plate.
All of the medium was removed from the wells and 0.3 ml of a
solution of 0.125 M Tris, 2% SDS and 10% glycerin was added.
The plates were allowed to stand for 15 min. and then 0.6 ml of
15   dH_2O was added to each well.

10.   The fluid in each well was resuspended with a
pipette and transferred to a glass test tube.   The tip was
ejected into the tube.   The tubes, including those containing
20   the input samples, were counted in a gamma counter.   CPMs bound
in each well were divided by the input CPMs for each sample to
determine the % bound.   Input CPMs were determined by counting
a 0.3 ml aliquot of each cell suspension described in step 8.
The results, shown in Fig. 6, indicate that the
25   monoclonal antibodies SNH-4 and CSLEX-1 specific for SLX
blocked the binding of HL-60 cells to GMP-140 on activated
platelets.   The monoclonal antibodies specific for Le^X (FH2 and
SH-1) also blocked this binding but to a lesser extent.   This
Example suggests that both SLX and Le^X may be ligands for GMP-
30   140, but that the SLX structure may be of a higher affinity for
GMP-140 than the Le^X structure.

EXAMPLE VI
Liposomes of Glycosphingolipids Block Binding of
SLX Cells to Activated Platelets
35   This Example demonstrates the ability of the
liposomes which contain SLX or SLX mimetics to block the
binding of SLX-expressing HL-60 cells and PMNs to platelets

66

which have been stimulated to express GMP-140 by treatment with
Thrombin. The assays generally followed the protocol described
in Larsen et al., Cell 63: 467-474 (1990), which is
incorporated herein by reference.

5   Materials:
             Glycosphingolipids were prepared as described in
Example IV. The platelets were prepared as described in
Example V, except that blocking of Fc receptors was not
performed. HL60 cells were prepared as described above.

10   PMNs were prepared from 50 ml of whole blood drawn
from volunteer donors into heparinized vacutainer tubes, which
were inverted to mix the blood. All steps were performed at
22-24 degrees C. Each 25 ml of blood was layered over 15 ml of
Mono-Poly Resolving Medium (Flow Labs). The tubes were

15   centrifuged at 800xg for 25 min followed by 1300xg for a
further 25 min.   The PMN layer was removed and placed in a
clean 50cc centrifuge tube. Thirty ml of Hanks Balanced Salt
Solution (Gibco) containing 20mM HEPES (Gibco) and 0.2%
glucose (Fisher) was added to each tube, which were then

20   centrifuged at 1900xg for 3 min. The PMNs were washed 3X in
the same buffer by centrifugation at 1900xg for 3 min. PMNs
were counted using a hemacytometer and resuspended to 2 x10^6/ml
and held at room temperature until use.

25   Procedure
             1.   20 ul of preparation of activated platelets were
placed in each of 28 1.5 ml eppendorf tubes (14 duplicate
samples).
             2.   20 ul of the diluted liposomes at 10 ug, 5 ug or

30   2 ug, or of the control buffers, were added to the appropriate
tube of each duplicate.
             3.   The platelets were incubated with the liposome
·preparations for 20 min. at room temp.
             4.   Neutrophils or HL-60 cells at 2 x 10^6 cells/ml

35   were each added to one set of liposome treated platelets.
20 ul of cell suspension were added to each tube.
             5.   The tubes were mixed and allowed to stand at
room temperature for 20 min. Then they were applied to a

hemacytometer and the cells were scored as positive (2 or more
platelets attached/cell) or negative (less than 2 platelets
attached/cell).

5   As shown in Fig. 7, liposomes containing selected
glycolipids having terminal sequences which contained SLX (S-
diLE^X, Table 1) dramatically inhibited adhesion of HL-60 cells
to activated platelets. Liposomes containing glycolipids with
Le^X (di-Le^X) or other related carbohydrate structures (Table 1)
exhibited minimal inhibition that was not dependent on the

10   structure of the carbohydrate group. Similar results were
obtained with PMN cell adhesion (Fig. 8). These experiments
show that biosynthetically produced SLX or similar SLX mimetic
compounds when formulated into liposomes compositions can serve
as therapeutic compounds for, e.g., the reduction of leukocyte

15   binding to platelets in inflammatory sites.

EXAMPLE VII

Oligosaccharide containing SLX

blocks binding of Neutrophils to platelets

In this example the ability of a minimal tetra-

20   saccharide SLX to inhibit GMP-140 adhesion was compared to that
of penta- and hexasaccharides containing SLX. Briefly,
platelets and neutrophils were isolated by the methods
described above. Platelets were activated with thrombin and
then incubated with dilutions of various oligosaccharides.

25   Neutrophils were added and the effect of the saccharides on the
adhesion of neutrophils to activated platelets was determined.
The oligosaccharides used were as follows: SLX(hexa),
NeuAcα2,3Galβ1,4 (Fucα1,3)GlcNacβ1,3 Galβ1,4Glc-O-CH$_2$CH$_2$SiMe$_3$
(the generous gift of Professor Hasegawa, Gifu University,

30   Japan), SLX(penta) NeuAcα2,3Galβ1,4(Fucα1,3)GlcNacβ1,3Galβ,
and SLX(tetra), NeuAcα2,3Galβ1,4 (Fucα1,3)GlcNAc.

· Procedure

35   1.   Platelets were isolated as described above and were
activated (2x10^8/ml) by incubation for 20 min at room
temperature with thrombin at a final concentration of
0.25U/ml.

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2.   Neutrophils were isolated by layering heparinized blood
      over Mono-Poly Resolving Medium (Ficoll-Hypaque-Flow
      Laboratories), followed by centrifugation for 25 min at
      2000rpm and then, a further 25 min at 2500rpm as described
5   above.

3.   For the assay, 20 μl of the platelet suspension (2×10^8/ml)
      was placed in an Eppendorf centrifuge tube. An equal
      volume of the oligosaccharide preparations at
10   concentrations from 200 μg/ml to 0.3 μg/ml, or of
      glycolipid-liposome preparations (prepared as described,
      above), at concentrations from 2 μg/ml to 0.25 μg/ml, was
      added and the tubes were allowed to stand at room
      temperature for 20 min. Twenty μl of the neutrophil
15   preparation (2×10^6/ml) was then added and the tubes were
      allowed to stand for a further 20 min at room temperature.

4.   Adhesion of activated platelets to the neutrophils was
      assessed microscopically. One hundred neutrophils were
20   evaluated. They were scored as positive if 2 or more
      platelets were attached and negative if less than 2
      platelets were bound. The percent of cells with 2 or more
      bound platelets was calculated.

25 The mean of the results of three identical experiments are
    shown in Table 2.

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TABLE 2

OLIGOSACCHARIDE	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
SLX (hexa)	1.8
SLX (penta)	2.2
SLX(tetra)	54.0
Le^X	43.0

As indicated in Table 2 above, approximately 20 times more of the SLX-tetrasaccharide is required for 50% inhibition of GMP-140 mediated binding of neutrophils to thrombin activated platelets than of the SLX-hexasaccharide. The amount of the tetrasaccharide required is approximately that needed for a similar degree of inhibition when the non-sialylated Le^X was used. The pentasaccharide gives 50% inhibition at concentrations similar to those required for 50% inhibition by the hexasaccharide which indicates that the minimal structure. for maximum inhibition is closer to a pentasaccharide.

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EXAMPLE VIII

Blocking adhesion using variant SLX structures

This example describes experiments testing various glycolipid structures on liposomes. In particular, SY2, a

5   sialylated polysaccharide in which the fucose instead of being attached to the ultimate GlcNAc as in SLX, is attached to the penultimate GLcNAc was tested. Platelets and neutrophils were isolated by the methods described above. Platelets were activated with thrombin and then incubated with dilutions of

10   various glycolipids embedded in liposomes prepared as described above. Neutrophils were added and the effect of the glycolipids on the adhesion of neutrophils to activated platelets was determined.

15   Structures of the various glycolipids examined are as follows: SDiY2, NeuGcα2,3Galβ1,4(Fucα1,3)GlcNacβ1,3Galβ1,4 (Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; SLX, NeuGcα2,3Galβ1,4 (Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; SY2, NeuGcα2,3Galβ1,4 GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; SH,

20   NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer; SPG, NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Cer.

        The results of two identical experiments are shown in Table 3.

25

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Table 3

GLYCOLIPID	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
SY2 (Exp 1)	0.325
SY2 (Exp 2)	0.345
SLX (hexa)	0.30
SdiY2	0.36
SPG	No Inh.
SH	No Inh.

These results show that SY2 inhibited GMP-140 mediated adhesion
of neutrophils to thrombin activated platelets equally as well
as did SLX and SDiY2.

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EXAMPLE IX

<u>Blocking adhesion using further variants of SLX</u>

The example demonstrates that the affinity of
sialylated Le^X(SLX) for GMP-140 is the same whether the
5 terminal sialic acid is in the form N-Acetyl neuraminate
(NeuAc) or N-Glycol neuraminate (NeuGc). All materials were
prepared as described above. Platelets and neutrophils were
isolated by the methods described. Platelets were activated
with thrombin and then incubated with dilutions of various
10 glycolipids contained in liposomes. Neutrophils were added and
the effect of the glycolipids on the adhesion of neutrophils to
activated platelets was determined.

The results of an experiment in which synthetic
15 SLX(NeuAc) and a preparation of SLX prepared by enzymatic
fucosylation of sialylparagloboside purified from bovine
erythrocytes SLX(NeuGc), were directly compared are shown in
Table 4.

20   Table 4

GLYCOLIPID	SOURCE	AMOUNT REQUIRED FOR 50% INHIBITION (μM)
SLX (NeuGc)	(Bovine Erythrocytes)	0.74
SLX (NeuAc)	(Synthetic)	0.67

These results show that SLX-hexasaccharide inhibited GMP-140
mediated adhesion of neutrophils to thrombin activated
35 platelets equally well whether the sialic acid was NeuAc or
NeuGc. This result indicates that either the N-acetyl or N-
glycollyl derivative of sialic acid are recognized by GMP-140.
Similar results have been obtained with ELAM-1.

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Various glycolipids were also tested in the same
assay. Structures of the glycolipids tested are as follows:
SLX(hexa), NeuGcα2,3Galβ1,4(Fucα1,3)GlcNacβ1,3Galβ1,4
4Glcβ1,1Ceramide; α2,3 SLX cer, NeuAcα2,3Galβ1,4(Fucα1,3)
5 GlcNAcβ1,3Galβ1,4Glcβ1,1Ceramide; α2,6 SLX cer,
NeuAcα2,6Galβ1,4(fucα1,3)GlcNacβ1,3Galβ1,4Glcβ1,1Ceramide; SH,
NeuGcα2,3Galβ1,4
GlcNAcβ1,3Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1,1Ceramide.

10   EXAMPLE X
                  Blocking adhesion using synthetic SLX
            This example demonstrates that synthetic SLX binds
      ELAM-1 and inhibits neutrophil adhesion to activated
      endothelium. This example also shows that the linkage of the
15   sialic acid affects binding to ELAM-1.

            Two synthetic compounds were prepared. One comprised
      sialic acid in an α2,3 linkage, as in naturally occurring SLX.
      The second comprised sialic acid in an α2,6 linkage, to examine
20   the importance of the nature of the linkage to receptor
      binding.

            Liposomes were prepared by adding 12 μl of absolute
      ETOH to each tube, warming briefly in a 50°C water bath and
25   sonicating for 2 min. 238 μl of warm phosphate buffered saline
      (PBS) was added slowly to each tube while sonicating and
      sonication was continued for a further 10 min. The final
      concentration of stock liposomes was 400 μg glycolipids/ml in
      5% ETOH/PBS.

30

Procedure

·1.   HUVECs, PMNs, and liposomes were prepared as described
      above.

35

2.   The stimulated HUVEC assay plate was removed from the
      incubator and the wells were washed two times with RPMI
      1640 containing 5 mg/ml bovine serum albumin (BSA).

3. Liposomes stocks were diluted in the HBSS/BSA buffer to
    make solutions equal to: 40 μg/ml, 30 μg/ml, 15 μg/ml, 7.5
    μg/ml, 3.75 μg/ml and 1.87 μg/ml. Similar dilutions were
    prepared from a control stock consisting of PBS-5% ETOH.

4. The medium was removed from the wells of the assay plate
    with a pasteur pipette, a few wells at a time.

5. 0.05 ml of each liposome suspension was added to duplicate
    wells on the stimulated assay plate. Control wells
    received the liposome buffer containing ethanol HBSS and
    BSA at the same concentrations as in the liposome
    containing wells. Control buffer was plated on
    unstimulated and stimulated HUVEC. Liposome containing
    samples were added to stimulated wells only.

6. The plates were incubated for 40 min at 37°C and then 50 μl
    of PMNs were added to the assay wells. The final
    concentration of cells was 5×10^5 well in 100 μl.

7. The assay plate was returned to the incubator (5% CO_2, 37
    °C) for 8 min.

8. Unbound cells were removed from the wells of the assay
    plates by systematic resuspension using a p200 multichannel
    pipette followed by addition and removal of 0.2 ml of
    medium.

9. All of the medium was removed from the wells and 50 μl of
    solubilization buffer was added. This consisted of citrate
    buffer (24.3 ml of 0.1 M Citric acid, 10.5 g/500 ml + 25.7
    ml of 0.2 M dibasic sodium phosphate, 14.2 g/500 ml and SQ
    H_2O to 100 ml) containing 0.1% NO-40 detergent.

10. The plate was incubated on a rotary shaker for 10 min and
    then 0.05 ml of OPDA solution [8 mg o-phenylene-diamine,
    Sigma cat# P-1526, 8 μl of 30% H_2O_2 and 10 ml of citrate
    buffer (as above)] was added to each well. The reaction

was allowed to develop for 15 min and then 25 μl of 4N
H$_2$SO$_4$ was added to each well to stop the reaction.

11. A reagent bulk was prepared by mixing 100 μl volumes of the
    solubilization buffer and the OPDA solution with 50 μl of
    4N H$_2$SO$_4$.

12. 100 μl of supernatant was removed from each of 2 wells and
    transferred to a flexible ELISA assay plate (Falcon). The
    plate scanned spectrophotometrically at 492 nM within 30
    min.

The results of the two experiments are presented in Table 5,
below.

Table 5

	Concentration	(2,6) SLex Mean	(2,3)SLex Mean
1	20 μg/ml	0.663	0.156
2	15 μg/ml	0.636	0.270
3	7.5 μg/ml	0.602	0.359
4	3.75 μg/ml	0.655	0.483
5	1.87 μg/ml	0.690	0.580
6	.47 μg/ml	0.695	0.642
7	0 μg/ml	0.710	0.716

	Concentration	Control +1L-1B Mean	Control -1L-1B Mean
1	20 μg/ml	0.657	0.010
2	15 μg/ml	0.740	0.010
3	7.5 μg/ml	0.658	0.013
4	3.75 μg/ml	0.698	0.009
5	1.87 μg/ml	0.725	0.014
6	.47 μg/ml	0.782	0.018
7	0 μg/ml	0.708	0.016

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These results show that liposomes containing synthetic α(2,3)SialylLe^X but not α(2,6)SialylLe^X inhibit neutrophil adhesion to activated endothelium in an ELAM-1 dependent binding assay. Thus, the α2,3 linkage of the sialic acid appears to be necessary for recognition by ELAM-1. In addition, the results show that a synthetically produced oligosaccharide, α(2,3)SialylLe^X, binds to ELAM-1 and blocks binding of neutrophils to activated endothelium. Similar results were found with GMP-140 and are presented in Figure 9. Similar results were found with GMP-140. See Figure 9. This compound or derivatives of this compound therefore constitute potential anti-inflammatory drug candidates.

EXAMPLE XI

Treatment of HL60 Cells with Endo-β-Galactosidase

This example describes experiments to determine whether the internal β-galactose-backbone sugar linkage of sialylated Le^X of HL60 cells was susceptible to cleavage by Endo-β-Galactosidase, an enzyme known to cleave an internal β-galactose linkage in polylactosaminyl structures, but not β-gal when GlaNAc is attached to mannose (core-type structures).

Procedure:

Platelets were isolated and activated with thrombin by the methods described above. Il-1β activated HUVEC were prepared as described above. Cultured HL60 cells were treated with endo-β-galactosidase as described below and the effect of enzyme treatment on the GMP-140 mediated adhesion of HL60 cells to activate platelets was determined.

Enzyme treatment of the HL60 cells was carried out as follows: 12.4 x10^6 cells were washed twice with Hanks Balanced Salt Solution containing 20mM HEPES and 0.2% glucose, followed by a single wash step in normal saline. The endo-β-galactosidase (0.1 Unit, ICN Chemicals, Inc., Irvine, CA) was dissolved in 200μl normal saline and 200μl sodium acetate buffer, pH 6.01. 200μl (containing 0.05U of enzyme) was added to 3 x 10^6 HL60 cells, and 200μl of the acetate buffer was added to a similar number of cells to be used as the buffer control. Both tubes were incubated at 37°C for 60 min. with

gentle shaking. The tubes were then cooled in ice and the
cells were washed three times in HBSS containing HEPES and
glucose and were then counted and suspended to $2x10^6$/ml.

5   For the GMP-140 assay, $20\mu l$ of Tyrode-HEPES buffer, pH
7.2 was placed in an Eppendorf tube. The same volume of
activated platelets ($2x10^8$/ml) and HL60 cells ($2x10^6$/ml) was
added and, after mixing, the tubes were allowed to stand at
room temperature for 20 min. Adhesion of platelets to the HL60
cells was assessed microscopically as described earlier for
10   adhesion of activated platelets to neutrophils.

       For the ELAM-1 assay, enzyme treatment of the HL-60
cells was performed as described above, except that the cells
were simultaneously labeled with ^{51}CR as previously described.
ELAM-1 mediated adhesion was arrested by incubating 2×10^5
15   treated or untreated cells with IL-1 activated HUVEC for 30
minutes at 4°C and then washing the plate with a pasteur
pipette.

       The results of these experiments indicated that treatment
of HL60 cells with Endo-β-Galactosidase inhibited their ability
20   to bind to (1) thrombin activated platelets by 87.5% and (2)
IL-1β activated HUVEC at 4°C by 70%. Thus, the minimal SLX-
containing tetrasaccharide ligand for GMP-140 is probably
attached to a lactose or polylactosaminyl structure rather than
a mannose.

25   EXAMPLE XII

                Fucosylated Polysaccharide blocks binding
                      of Neutrophils to Platelets

30   In this example the ability of a fucosylated
polysaccharide to inhibit GMP-140 mediated adhesion was
compared to that of the non-fucosylated polysaccharide, a
hexasaccharide SLX and Le^X. Briefly, platelets and neutrophils
were isolated by the methods described above. Platelets were
35   activated with thrombin and then incubated with dilutions of
various oligosaccharides. Neutrophils were added and the
effect of the saccharides on the adhesion of neutrophils to
activated platelets was determined. The oligosaccharides used
were as follows: Native polysaccharide and its fucosylated
40   derivative (the preparation of both is described, below); SLX

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hexasaccharide, LNF III (Le^X) and LNF I (the structures are
described above).

The conversion of a polysaccharide which contains the
linear core structure of SLX into a polyvalent SLX containing
5 polysaccharide was achieved by enzymatic fucosylation. The
native polysaccharide type Ia was obtained from Group B
Streptococcus as described by Jennings et al., Biochem. _22_
1258-1263 (1983) which is incorporated herein by reference.
The appropriate bacterial strains are deposited with the
10 American Type Culture Collection and have Deposit Nos. 12400,
31574, 12401, and 31575.

To prepare the fucosylated polysaccharide, the native
type Ia polysaccharide 1 mg. was dissolved in a mixture of 6 μL
of 1 M manganese chloride, guanosine 5'-diphosphate β-L-fucose
15 with a radiolabelled tracer (specific activity 1.82 x 10^6
cpm/μmol), 0.9 μmoles in water 90 μL and water 137 μL. To this
was added 100 μL solution of 3/4 fucosyl transferase isolated
from human milk as previously described by Prieels et al.,
J.Biol.Chem. _256_ 10456-10463 (1981) which is incorporated
20 herein by reference.

The reaction mixture was concentrated against a
membrane (100K cut off) several times with water and the
retentate lyophylized to give a powder. This solid was
dissolved in water and passed through a weak cation exchange
25 column to remove any remaining protein. The radioactive
fractions containing the fucolsylated polysaccharide were
collected and lyophilized. Approximately fifty of the
available side chains were fucosylated as measured by the
incorporation of the radiolabel.
30 Procedure:

Platelets were isolated as described above and were
activated (2x10^8/ml) by incubation for 20 min at room
·temperature with thrombin at a final concentration of 0.25U/ml.

Neutrophils were isolated by layering heparinized
35 blood over Mono-Poly Resolving Medium (Ficoll-Hypaque, Flow
Laboratories), followed by centrifugation for 25 min at 2000rpm
and then, a further 25 min at 2500rpm as described above.

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For the assay, 20 µl of the platelet suspension
(2×10^8/ml) was placed in an Eppendorf centrifuge tube. An
equal volume of the oligosaccharide preparations at ·
concentrations from 500µg/ml to 2.0µg/ml was added and the
5   tubes were allowed to stand at room temperature for 20 min.
Twenty µl of the neutrophil preparation (2×10^6/ml) was then
added and the tubes were allowed to stand for a further 20 min
at room temperature.

     Adhesion of activated platelets to the neutrophils was
10   assessed microscopically. One hundred neutrophils were
evaluated. They were scored as positive if 2 or more platelets
were attached and negative if less than 2 platelets were bound.
The percent of cells with 2 or more bound platelets was
calculated.

15   As shown in Table 6, the fucosylated polysaccharide
very efficiently inhibited GMP-140 mediated binding of
neutrophils to thrombin activated platelets- 50% inhibition was
achieved with less than 1µg/ml. This compared to 20µg/ml which
was required of the native polysaccharide and 8µg/ml of the SLX
20   hexasaccharide for a similar degree of inhibition.

TABLE 6

OLIGOSACCHARIDE	AMOUNT REQUIRED FOR 50% INHIBITION (µM)
Native Polysaccharide	15
Fucosylated Polysaccharide	0.7
SLe^X Hexasaccharide	8
LNF III (Le^X)	35
· LNF I	No Inhibition

40

80

EXAMPLE XIII

Protection of Rats from Endotoxic Shock (Lethality) by a Monoclonal Antibody to LAM-2

5   This example demonstrates the efficacy of mAb P6E2 (a
murine IgG3k, functional anti-human ELAM-1 mAb, described in
copending application U.S.S.N. 07/645,878, which is
incorporated herein by reference) in an animal model of
lipopolysaccharide-induced death. A rat system was chosen
10   because P6E2 has been shown to cross-react with the rat
equivalent to ELAM-1.

Materials and Methods

       LPS from *E. coli* 0111:B4 (Sigma) was prepared fresh
15   from a single lot one day prior to use by dissolving in
sterile, pyrogen-free saline at a concentration of 5 mg/ml.
The solution was sonicated on ice for 30 seconds using a
Tekmark Sonic disrupter. Just prior to use, the material was
sonicated a second time for 30 seconds.
20   Female Lewis rats' weighing 200 g (+- 10g) were
purchased from Charles River Breeding Labs and held for at
least 7 days after receipt (for adaptation). Groups
(10 animals) were used, unless otherwise noted. All reagents
were injected parenterally via tail vein. As negative
25   controls, animals received either sterile, LPS-free saline, or
a murine lgG3k myeloma protein (J606, low pyrogen - < 2 ng/mg
protein).

Results

30   The P6E2 dose/schedule protocols were arrived at
empirically from the pharmacokinetic data we obtained with P6E2
prophylactically administered to rats. A "minimal" LD_{100} dose
was empirically determined to be 7.5 mg/kg for these rats.
       In one experiment, rats were treated with 10 mg/kg one hour
35   before the LPS challenge. A boost was administered 3 hours
after the challenge. 4/10 treated animals survived the LPS
challenge. In contrast, all 10 (saline-injected) controls
died. At the twenty-four hour observation period, the

survivors showed few of the clinical signs characteristic of
LPS-treated animals.

We next tried doses of P6E2 which were (1) 2-fold higher
and (2) an order of magnitude lower than the 10 mg/kg dose used
in the first experiment. The results show that P6E2 had a
significant effect: 80% of the animals survived at the 10
mg/kg dose (Figure 10). Note that one animal survived in the
saline control group - suggesting that this group of animals
was not "hit" as hard.

Another study was performed to demonstrate the
therapeutic value of P6E2. Animals received the 10 mg/kg iv
bolus dose of P6E2 at 1 hour before or 2, 4 or 6 hours after
the LPS challenge.

Once again, 1 of 10 animals survived in the saline-
treated group, as well as in a group treated with 10 mg/kg J606
myeloma protein at T=-60 minutes (Figure 11). P6E2 had a
protective effect even when administered 2 or 4 hours after
LPS.

Conclusion

The protection seen with Cytel mAb P6E2 demonstrates
of the importance of ELAM-1 in an animal model of a lethal
disease.

EXAMPLE XIV

Rolling of SLex Liposomes on IL-1 Activated Rabbit Endothelial
Cells.

Purpose

This example demonstrates that sialylated Lewis x
(SLX) and sialoparagloboside (SPG) isolated from biological
sources and incorporated into liposomes will "roll" along
activated rabbit venules.

"Rolling" is an early intercellular interaction between
leukocytes and the endothelial cell wall. A leukocyte will
"roll" on endothelial cells. The leukocyte will then either
(1) be released back into circulation, or (2) adhere to the
endothelial cell, and begin the early events that culminate in
inflammation. Selectins have been implicated in the cell-cell

interactions of "rolling." von Adrian, et al., Proc. Nat'l
Acad. Sci., _____:_____ (1991).

Materials

5   Liposomes containing the glycosphingolipids were
formed as follows: 50 ug of glycolipid was added to 150 ug
phosphatidylcholine (Sigma, egg yolk), 250 ug cholesterol
(Sigma) and 1 mM carboxyfluorescein (Sigma) in
chloroform:methanol (2:1) and the whole solution evaporated to
10   dryness in glass screw cap tubes. Liposomes were prepared by
adding 12.5 ul of absolute ethanol to each tube, warming
briefly in a water bath and sonicating for 2 minutes 238 ul of
warm PBS was added slowly to each tube while sonicating and
sonication was continued for a total of 10 minutes. The
15   liposomes were brought to 1 ml with PBS and centrifuged at
14,000 rpm for 2 minutes to remove excess EtOH and
carboxyflourescein. The supernatant was discarded and the
liposomes resuspended in 1 ml PBS, counted on a hemacytometer
and adjusted to 5×10^6 liposomes per ml. One ml is injected
20   into the rabbit, activated 4 hours earlier with IL-1.
        Rolling was observed using intravital microscopy as
described by von Adrian et al. IL-1 activated rabbit mesentery
was exteriorized and spread on a 37°C heated glass window over
a microscopic stage. The mesentery was bathed with 36.5°C
25   saline solution equilibrated with 5% CO_2 in N_2. Liposome-
endothelial interactions were observed with an intravital
microscope with a 50X salt water immersion objective.
Liposomes were visualized by exposure to fluorescent light.
The liposomes in a segment of selected mesentery venule (20-
30   40μm in diameter) were counted for one minute.

Results

            Liposome Rolling on IL-1 Activated Rabbit Cells

material injected	rolling
35 human neutrophils	4 (maximum amount of rolling)
SLex liposomes	2
SPG liposomes	0 (no rolling)

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Conclusion

In addition to LAM-1 (von Andrian, et al.), ELAM-1 and it's ligand appear to be involved in the process of leukocyte
5   margination or "rolling".

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of
10   clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound comprising a moiety which selectively binds a selectin receptor, the moiety

5   having the formula:

R_1-Galβ1,4(Fucα1,3)GlcNAc-$(R_2)_a$

wherein R_1 is an oligosaccharide or R_3-R_4-C(CO$_2$H)-,

wherein R_3 and R_4 are the same or different and are -H, - C1-C8 alkyl, - hydroxyl C1-C8 alkyl, -aryl

10 C1-C8 alkyl, or -alkoxy C1-C8 alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

2. A composition of claim 1, wherein the compound is

15 a biomolecule.

3. A composition of claim 1, wherein the biomolecule is an oligosaccharide, an oligopeptide, a protein, or a lipid.

20 4. A composition of claim 1, wherein R_3 and R_4 are connected to form a 4-8 membered ring.

5. A composition of claim 4, wherein the 4-8 membered ring is a monosaccharide.

25

6. A composition of claim 5, wherein the monosaccharide is a sialic acid.

7. A composition of claim 6, wherein the sialic acid

30 is NeuAcα2,3 or NeuGcα2,3.

8. A composition of claim 1, wherein the oligosaccharide is NeuAcα2,3Galβ1,4GlcNAcβ1,3 or NeuGcα2,3Galβ1,4GlcNAcβ1,3.

35

9. A composition of claim 1, wherein the compound is a polysaccharide.

10.   A composition of claim 9, wherein the polysaccharide comprises a repeat unit having the formula:

NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4 →
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$|β1,4
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$. Glc
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$↑

11.   A composition of claim 9, wherein the polysaccharide comprises a repeat unit having the formula:

$\quad\quad\quad\quad$↓
NeuAcα2,3Galβ1,4(Fucα1,3)GLcNAcβ1,3Galβ1,4Glcβ1,3Glcβ1,2→
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$|β1,6
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$Gal

12.   A composition of claim 9, wherein the polysaccharide comprises a repeat unit having the formula:

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$↓
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$Glc
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$|β1,6
NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4 →
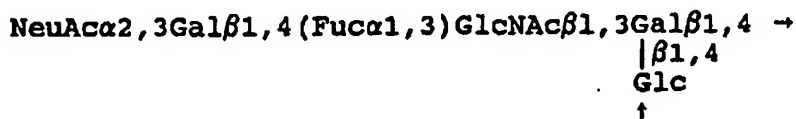
13.   A composition of claim 9, wherein the polysaccharide is a fucosylated type Ia polysaccharide of Group B streptococcus.

14.   A composition of claim 9, wherein the polysaccharide is a type II or type III polysaccharide of Group B streptococcus.

15.   A composition of claim 9, wherein the polysaccharide has molecular weight between about 5,000 and 300,000 daltons.

16.   A composition of claim 9, wherein the polysaccharide comprises between about 5 and about 200 fucosylated repeat units.

17.   A composition of claim 16, wherein the polysaccharide comprises between about 25 and about 100 fucosylated repeat units.

18.   A composition of claim 1, wherein the biomolecule is a sphingolipid.

19.   A composition of claim 18, wherein the
5   biomolecule is a ganglioside.

20.   A composition of claim 1, wherein the selectin receptor is expressed on a vascular endothelial cell or a platelet.
10

21.   A composition of claim 20, wherein the selectin receptor is ELAM-1 or GMP-140.

22.   A pharmaceutical composition which comprises a
15   pharmaceutically acceptable carrier and a liposome having a compound which selectively binds a selectin receptor.

23.   A composition of claim 22, wherein the liposome encapsulates an anti-inflammatory chemotherapeutic agent.
20

24.   A composition of claim 23, wherein the anti-inflammatory agent is cyclosporin A, indomethacin, naproxen, FK-506, or mycophenolic acid.

25   25.   A composition of claim 22, wherein the compound has the formula

R_1-Galβ1,4(Fucα1,3)GlcNAc-$(R_2)_a$-X
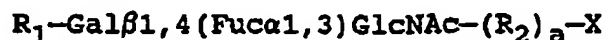
wherein R_1 is selected from the group consisting of NeuAcα2,3, NeuGcα2,3, NeuAcα2,3Galβ1,4GlcNAcβ1,3,
30   and NeuGcα2,3Galβ1,4GlcNAcβ1,3;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1; and

wherein X is a protein or lipid.

35   26.   A composition of claim 25, wherein X is a glycoprotein having a molecular weight between 40,000 and about 250,000 daltons.

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27. A composition of claim 25, wherein X is a
glycolipid having a molecular weight between about 600 and
about 4,000 daltons.

5   28. A composition of claim 22, wherein the compound
comprises a moiety having the formula:
           NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc—(R)$_a$—,
           NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAc—(R)$_a$—,   and
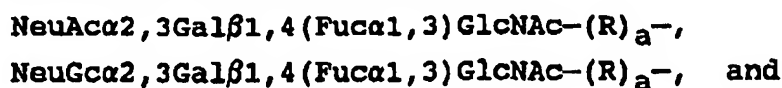
10   NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAc—(R)$_a$—;
           wherein R is β1,3Gal, α1,2Man, or α1,6GalNAc and a is
       0 or 1.

           29. A composition of claim 22, wherein the selectin
15   receptor is expressed on a vascular endothelial cell or a
platelet.

           30. A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and a compound which
20   selectively binds a selectin receptor, the compound having the
formula:
           R$_1$—Galβ1,4(Fucα1,3)GlcNAc—(R$_2$)$_a$—X
           wherein R$_1$ is an oligosaccharide or R$_3$—R$_4$—C(CO$_2$H)—,
                wherein R$_3$ and R$_4$ are the same or different and
25   are —H, —lower alkyl (C1-C8), —hydroxyl lower alkyl,
           —aryl alkyl, or —alkoxy alkyl;
                wherein R$_2$ is β1,3Gal, α1,2Man, or α1,6GalNAc and a is
       0 or 1; and
                wherein X is selected from the group consisting of —H,
30   —OH, —NH$_3$, —NHR$_3$, —NR$_3$R$_4$, —OR$_3$, —OAryl, —OAlkylAryl,
           —OArylAlkyl, —R$_3$, —Aryl, —ArylAlkyl, and —AlkylAryl,
           wherein R$_3$ and R$_4$ are the same or different and are C$_1$–C$_{20}$
           alkyl.
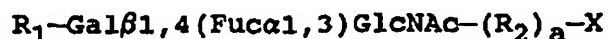
35   31. A composition of claim 30, wherein the compound
has the formula:
           NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc—(R)$_a$,
           NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAc—(R)$_a$,   and
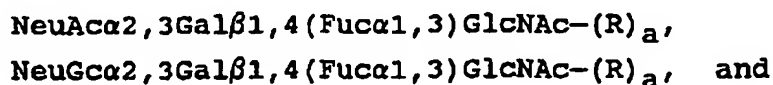
NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAc-(R)$_a$;
    wherein R is β1,3Gal, α1,2Man, or α1,6GalNac and a is
0 or 1.

5   32. A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and a compound having two
or more repeat units capable of selectively binding a selectin
receptor, the repeat units comprising a selectin-binding moiety
and being linked by a linker moiety, each repeat unit having
10   the formula:

R_1-Galβ1,4(Fucα1,3)GlcNAc-(R_2)$_a$
    wherein R_1 is an oligosaccharide or R_3-R_4-C(CO$_2$H)-,
        wherein R_3 and R_4 are the same or different and
are -H, - C1-C8 alkyl, - hydroxyl C1-C8 alkyl, -aryl
15   C1-C8 alkyl, or -alkoxy C1-C8 alkyl;
    wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is
0 or 1; and
    wherein X is the linker moiety.

20   33. A composition of claim 32, wherein the linker
moiety has the formula:
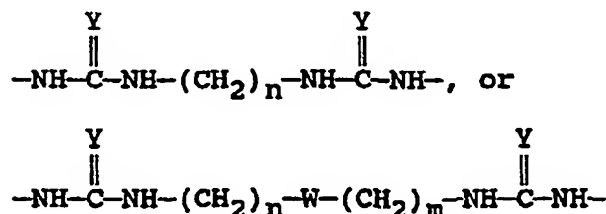
$$-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-(CH_2)_n-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-, \text{ or}$$

$$-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-(CH_2)_n-W-(CH_2)_m-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-$$

30   wherein, n and m are the same or different and are integers
from 2 to 12; Y is O or S; and W is O, S, or NH.

        34. A composition of claim 32, wherein the linker
moiety is 5- to 14-membered ring having two substituents, each
35   substituent having the formula

$$-NH-\overset{\overset{\displaystyle Y}{\|}}{C}-NH-$$
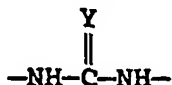
40   wherein, Y is O or S; and
    the substituents being in a cis- or trans-relationship.

35. A composition of claim 34, wherein the
substituents are in a 1,2 to 1,(p/2)+1 arrangement, wherein p
is an integer from 5 to 14 and corresponds to the size of the
ring.

36. A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and a heterocyclic compound
having two nitrogen atoms and two selectin-binding moieties,
each moiety being linked to one of the nitrogen atoms and
having the formula:
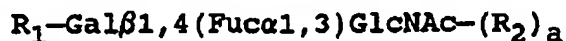
$$R_1-Gal\beta1,4(Fuc\alpha1,3)GlcNAc-(R_2)_a$$
wherein R_1 is an oligosaccharide or $R_3-R_4-C(CO_2H)-$,
wherein R_3 and R_4 are the same or different and
are $-H$, $- C1-C8$ alkyl, $-$ hydroxyl $C1-C8$ alkyl, $-$aryl
$C1-C8$ alkyl, or $-$alkoxy $C1-C8$ alkyl;
wherein R_2 is $\beta1,3Gal$, $\alpha1,2Man$, or $\alpha1,6GalNac$ and a is
0 or 1.

37. A composition of claim 36, wherein the
heterocyclic compound is piperazine or homopiperazine.

38. A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and an amino acid linked to
a selectin-binding oligosaccharide moiety selected from the
group consisting of
$$NeuAc\alpha2,3Gal\beta1,4(Fuc\alpha1,3)GlcNAc-(R)_a-,$$
$$NeuGc\alpha2,3Gal\beta1,4(Fuc\alpha1,3)GlcNAc-(R)_a-, \quad and$$
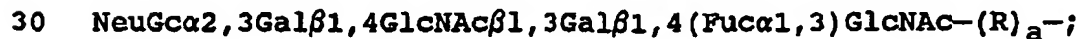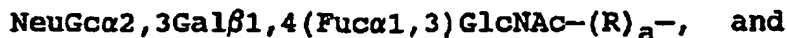
$$NeuGc\alpha2,3Gal\beta1,4GlcNAc\beta1,3Gal\beta1,4(Fuc\alpha1,3)GlcNAc-(R)_a-;$$
wherein R is $\beta1,3Gal$, $\alpha1,2Man$, or $\alpha1,6GalNac$ and a is
0 or 1.

39. A composition of claim 38, wherein the amino acid
is lysine, homolysine, ornithine, diaminobutyric acid,
asparagine or diaminopropionic acid.

40. A composition of claim 38, wherein the amino acid is incorporated in an oligopeptide.

41. A composition of claim 40, wherein the oligopeptide comprises one or more of the following: lysine, homolysine, ornithine, diaminobutyric acid, asparagine or diaminopropionic acid.

42. A composition of claim 41, wherein the oligopeptide further comprises one or more of the following: alanine, tyrosine or radioiodinated tyrosine.

43. A composition of claim 40, wherein the oligopeptide comprises, in a direction from the N-terminus to the C-terminus,

$$
\begin{array}{cc}
L & L \\
| & | \\
\end{array}
$$
L–Lys–Lys or

$$
\begin{array}{ccc}
L & L & L \\
| & | & | \\
\end{array}
$$
L–Lys–R_1–Lys–R_2–Lys–Ala,

wherein R_1 and R_2 are the same or different and are any amino acid residue and L is the oligosaccharide moiety.

44. A pharmaceutical composition for treating an inflammatory condition, the composition comprising a pharmaceutically acceptable carrier and an immunoglobulin capable of selectively binding an oligosaccharide ligand recognized by a selectin cell surface receptor, the immunoglobulin being present in an amount sufficient to treat the condition.

45. A composition of claim 44, wherein the ligand is selected from the group consisting of

NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc–(R)$_a$–,

NeuGcα2,3Galβ1,4(Fucα1,3)GlcNAc–(R)$_a$–, and

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NeuGcα2,3Galβ1,4GlcNAcβ1,3Galβ1,4(Fucα1,3)GlcNAc-(R)$_a$-;
wherein R is β1,3Gal, α1,2Man, or α1,6GalNac and a is
0 or 1.

5   46.   A composition of claim 44, wherein the
oligosaccharide moiety is expressed by a leukocyte.

        47.   A composition of claim 44, wherein the selectin
receptor is expressed by a vascular endothelial cell or a
10   platelet.

        48.   A composition of claim 44, wherein the selectin
receptor is ELAM-1 or GMP-140.

15   49.   A composition of claim 44, wherein the
composition is in unit dosage form.

        50.   A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and a compound comprising a
20   moiety which selectively binds a selectin receptor, the moiety
having the formula:
        R$_1$-Galβ1,4(R$_2$)GlcNac-(R$_3$)$_a$-,
        wherein R$_1$ is NeuAcα2,3, NeuGcα2,3, NeuAcα2,3,
        Galβ1,4GlcNAcβ1,3, or NeuGcα2,3Galβ1,4GlcNacβ1,3;
25   wherein R$_2$ is Fucα1,3, Araα1,3, (R,S)-5-alkyl-Araα1,3
        and (R,S)-5-aryl-Araα1,3; and
        wherein R$_3$ is 1,3βGal, 1,2αMan, or 1,6αGalNAc and a is
        0 or 1.

30   51.   A composition of claim 51, wherein the compound
is a biomolecule.

        52.   A composition of claim 51, wherein the moiety
binds a selectin receptor expressed on a vascular endothelial
35   cell or a platelet.

        53.   A composition of claim 51, wherein the selectin
receptor is ELAM-1 or GMP-140.

54. A method for inhibiting selectin-mediated
intercellular adhesion in a patient, the method comprising
administering to the patient a therapeutically effective dose
of a pharmaceutical composition comprising a pharmaceutically
5   acceptable carrier and a compound which selectively binds a
selectin receptor.

55.   A method of claim 55, wherein the compound
comprises a moiety which selectively binds a selectin receptor,
10 the moiety having the formula:

R_1–Galβ1,4(Fucα1,3)GlcNAc–$(R_2)_a$
wherein R_1 is an oligosaccharide or R_3–R_4–C(CO_2H)–,
wherein R_3 and R_4 are the same or different and
are –H, –lower alkyl (C1-C8), –hydroxyl lower alkyl,
15   –aryl alkyl, or –alkoxy alkyl;
wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is
0 or 1.

56. A method of claim 55, wherein the compound is a
20 biomolecule.

57. A method of claim 55, wherein the intercellular
adhesion is associated with an inflammatory condition.

25   58. A method of claim 58, wherein the inflammatory
condition is septic shock.

59. A method of claim 58, wherein the inflammatory
condition is acute respiratory distress syndrome or wound
30 associated sepsis.

60. A method of claim 55, wherein the intercellular
· adhesion is associated with metastasis.

35   61. A method of claim 55, wherein the selectin
receptor mediates adhesion of a leukocyte, monocyte or
neutrophil to an endothelial cell.

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62.   A method of claim 55, wherein the selectin receptor is ELAM-1 or GMP-140.

63.   A method of claim 55, wherein the compound is embedded in a liposome.

64.   A method of claim 55, the compound is a polysaccharide.

65.   A method of treating an inflammatory disease process mediated by a selectin receptor in a patient, the method comprising administering to the patient a therapeutically effective dose of a compound which selectively binds the receptor, the compound having the formula:

R_1-Galβ1,4(Fucα1,3)GlcNAc-$(R_2)_a$-X

wherein R_1 is an oligosaccharide or R_3-R_4-C(CO$_2$H)-,

wherein R_3 and R_4 are the same or different and are -H, -lower alkyl (C1-C8), -hydroxyl lower alkyl, -aryl alkyl, or -alkoxy alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1; and

wherein X is a biomolecule.

66.   A composition of claim 66, wherein X is an oligosaccharide, an oligopeptide, a protein, or a lipid.

67.   A method of claim 66, wherein the selectin receptor is ELAM-1 or GMP-140.

68.   A method of assaying a test compound for the ability to inhibit selectin-mediated cellular adhesion, the method comprising the steps of:

contacting the test compound with a selectin receptor and an isolated selectin-binding agent; and

detecting the ability of the test compound to inhibit binding between the receptor and the agent.

69. A method of claim 69, wherein the agent comprises an SLX moiety, or an SLX mimetic.

70. A method of claim 69, wherein the receptor or the agent are immobilized on a solid surface.

71. A method of claim 69, wherein the test compound is an oligosaccharide or a glycoconjugate.

72. A pharmaceutical composition comprising a compound capable of blocking selectin-mediated cellular adhesion identified by the method of claim 69.

73. A method for preparing a compound comprising an oligosaccharide moiety capable of selectively binding a selectin receptor, the method comprising fucosylating a polysaccharide comprising a sequence having the formula:

$$R-Gal\beta 1,4GlcNAc-$$
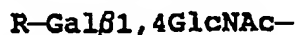
wherein R is a sialic acid.

74. A method of claim 74, wherein the step of fucosylating is carried out using a $\alpha 1,3$ fucosyltransferase.

75. A method of claim 74, wherein the polysaccharide is a type Ia polysaccharide of Group B streptococcus.

76. A method of claim 74, wherein the polysaccharide is a type II or type III polysaccharide of Group B streptococcus.

77. A method for preparing a compound comprising a plurality of moieties capable of selectively binding a selectin receptor, the method comprising linking the moieties together using a linker moiety.

78. A method of claim 78, wherein the moieties are selected from the group consisting of
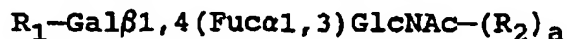
$$R_1-Gal\beta 1,4(Fuc\alpha 1,3)GlcNAc-(R_2)_a$$

wherein R_1 is an oligosaccharide or R_3–R_4–$C(CO_2H)$–,

wherein R_3 and R_4 are the same or different and are –H, – C1-C8 alkyl, – hydroxyl C1-C8 alkyl, –aryl C1-C8 alkyl, or –alkoxy C1-C8 alkyl;

wherein R_2 is β1,3Gal, α1,2Man, or α1,6GalNac and a is 0 or 1.

79. A method of claim 78, wherein the linker moiety has the formula:
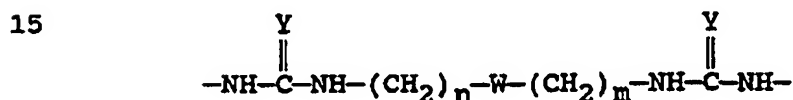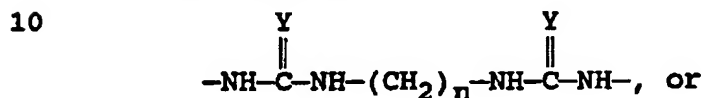
$$-NH-\overset{\overset{Y}{\|}}{C}-NH-(CH_2)_n-NH-\overset{\overset{Y}{\|}}{C}-NH-, \text{ or}$$

$$-NH-\overset{\overset{Y}{\|}}{C}-NH-(CH_2)_n-W-(CH_2)_m-NH-\overset{\overset{Y}{\|}}{C}-NH-$$

wherein, n and m are the same or different and are integers from 2 to 12; Y is O or S; and W is O, S, or NH.

80.   A method of claim 78, wherein the linker moiety is 5- to 14-membered ring having two substituents, each substituent having the formula

$$-NH-\overset{\overset{Y}{\|}}{C}-NH-$$
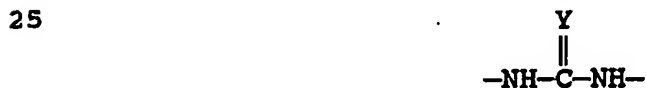
wherein, Y is O or S; and

the substituents being in a cis- or trans-relationship.

81. A method of claim 78, wherein the substituents are in a 1,2 to 1,(p/2)+1 arrangement, wherein p is an integer from 5 to 14 and corresponds to the size of the ring.

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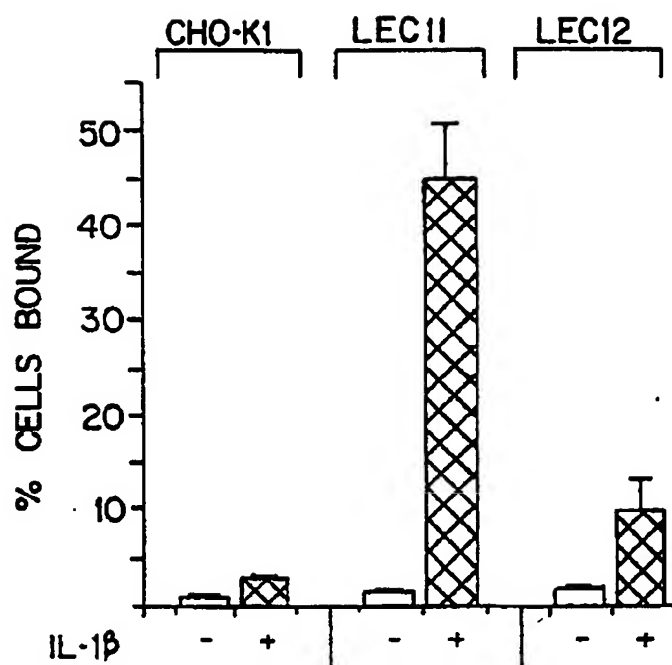
FIG. 1.

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mAbs BLOCK BINDING OF HL-60
TO STIMULATED HUVEC AT 37°C.
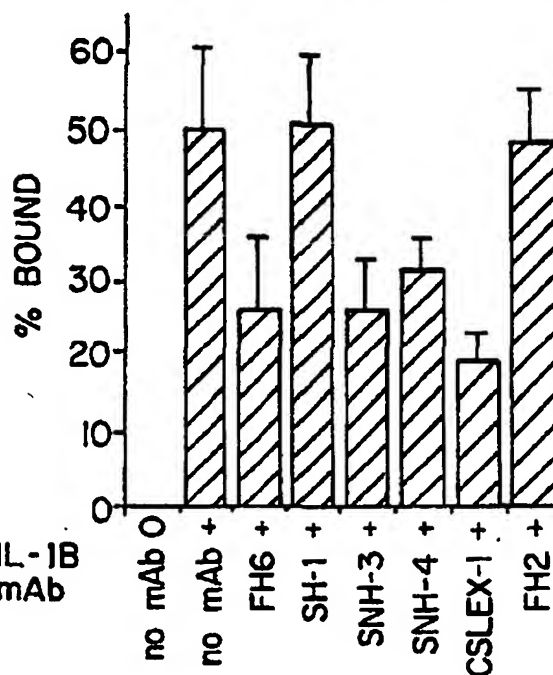
FIG. 2A.

mAb BLOCK BINDING OF HL-60
TO STIMULATED HUVEC AT 4°C.

FIG. 2B.

INHIBITION OF LEC11 BINDING TO
ACTIVATED ENDOTHELLUM BY mAb

FIG. 3A.

INHIBITION OF LEC12 BINDING TO
ACTIVATED ENDOTHELLUM BY mAb

FIG. 3B.

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FIG. 4.

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FIG. 5.

INHIBITION OF PLATELET ADHESION BY mAb.

FIG. 6.

FIG. 7.

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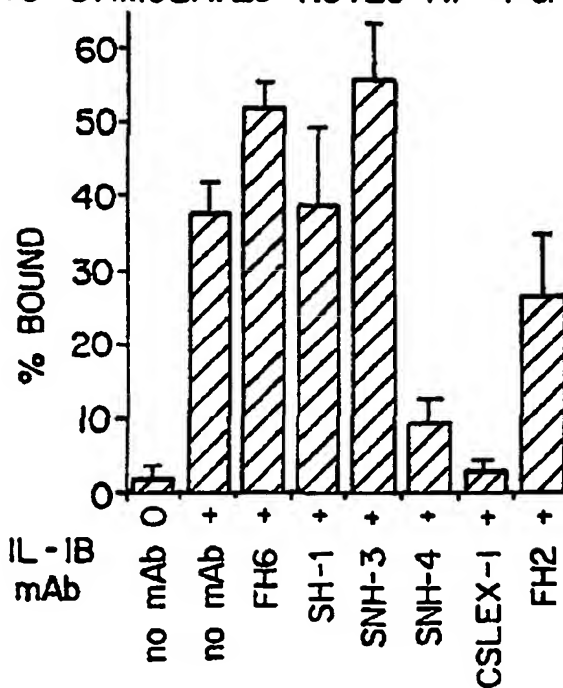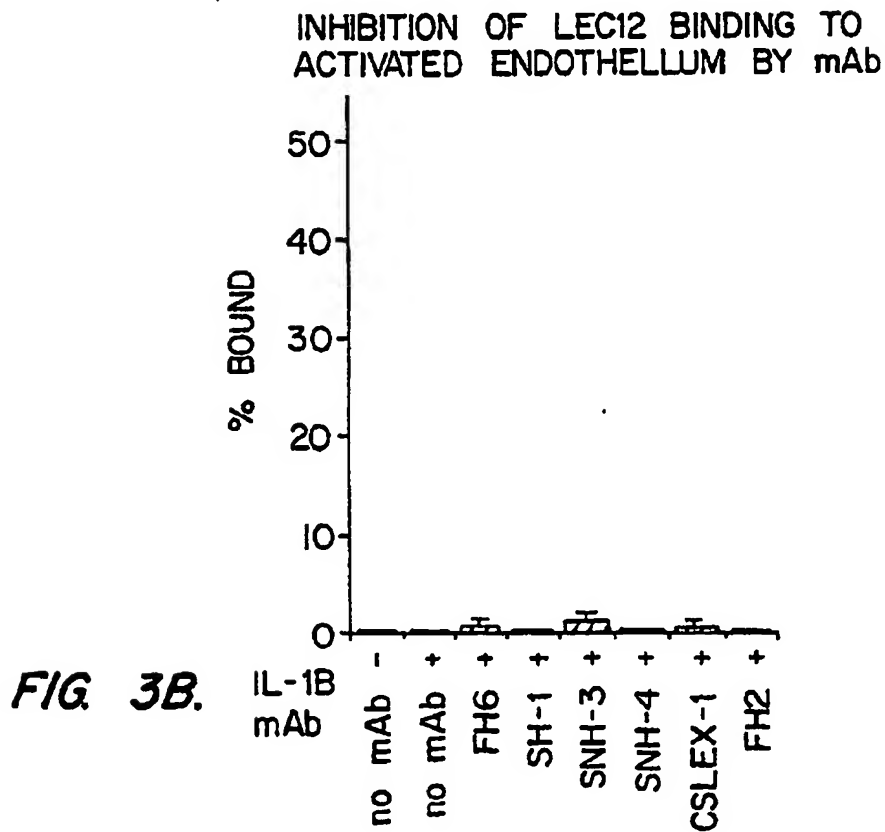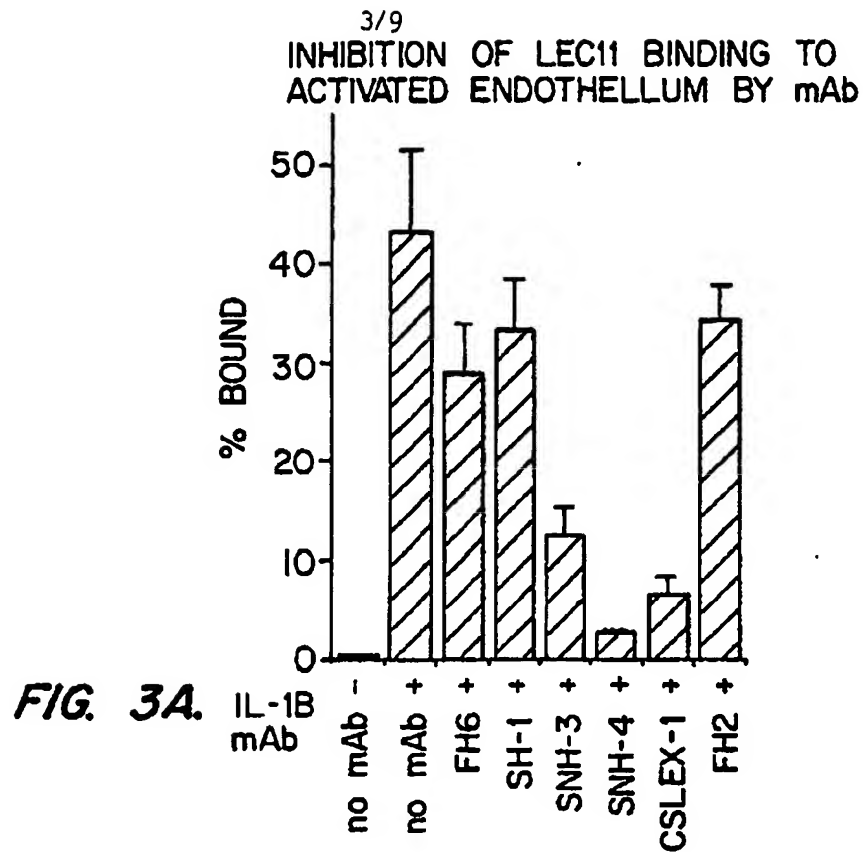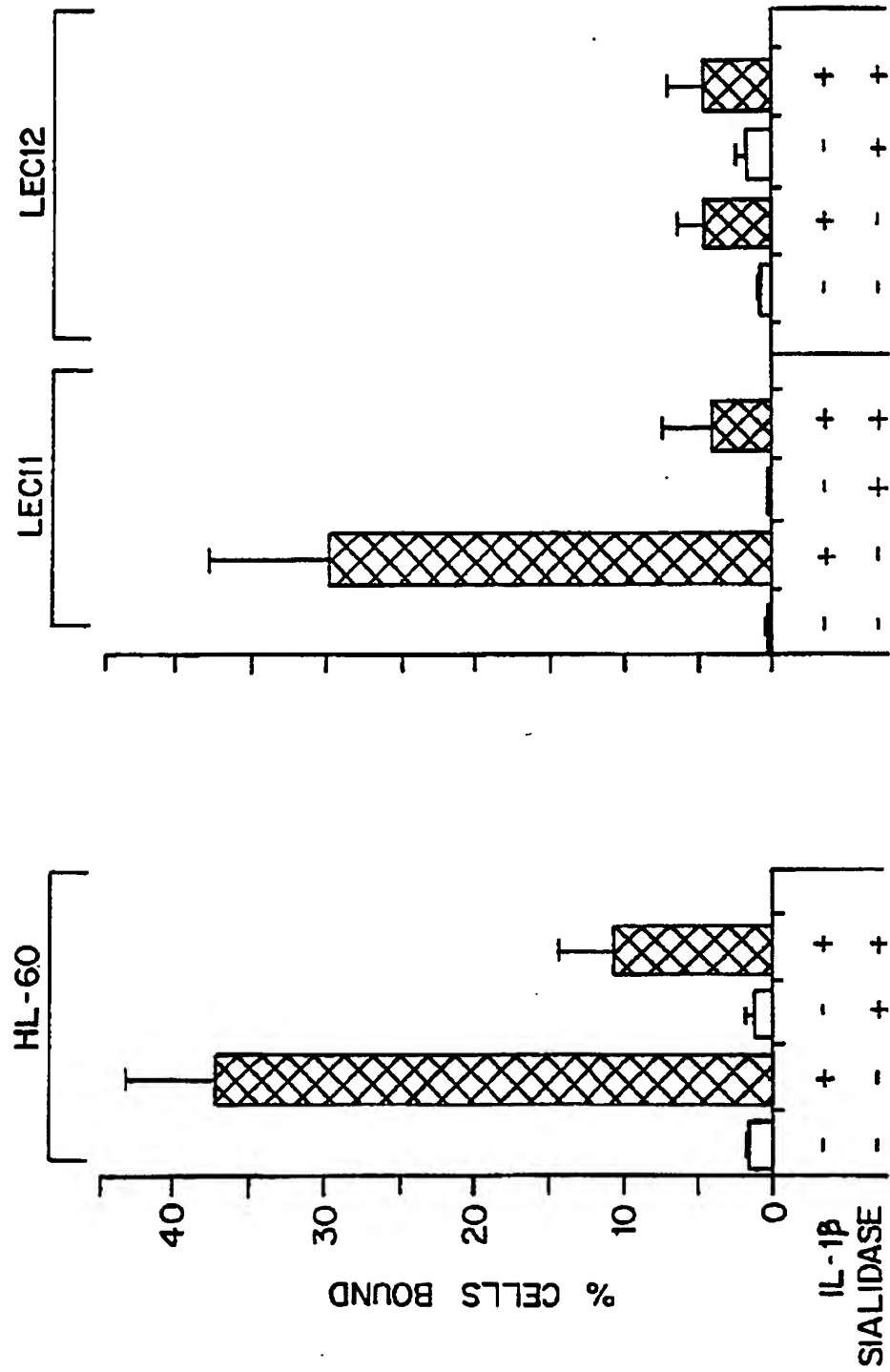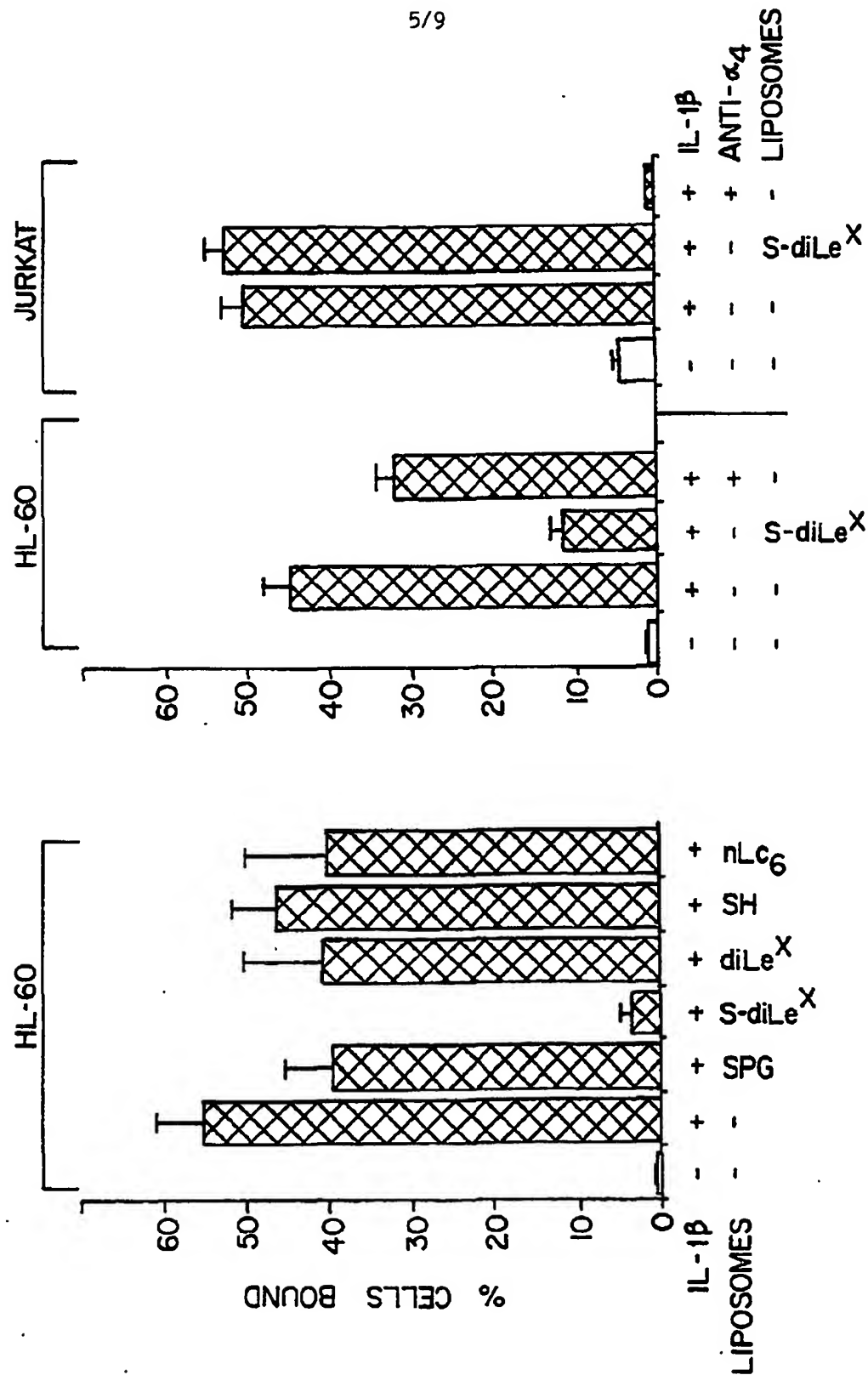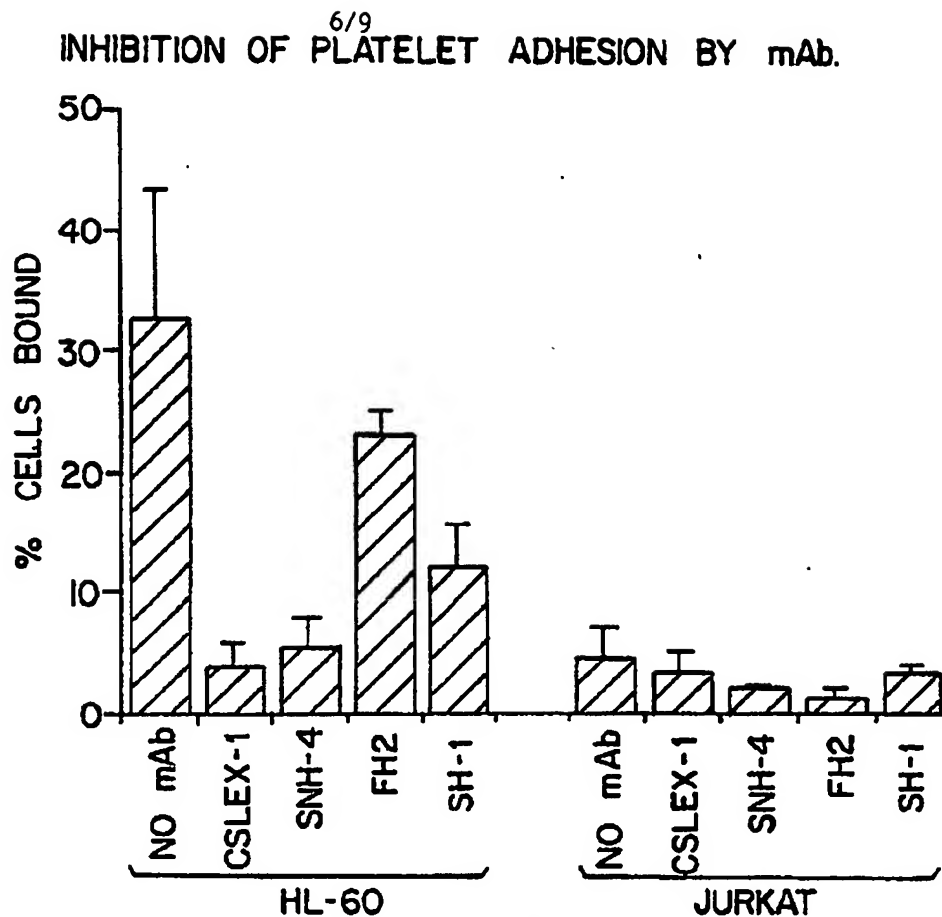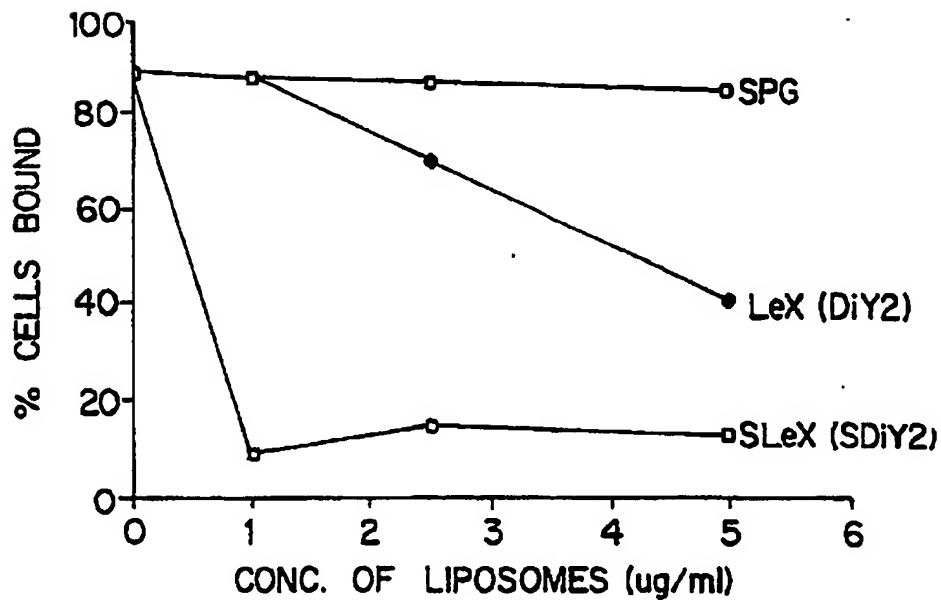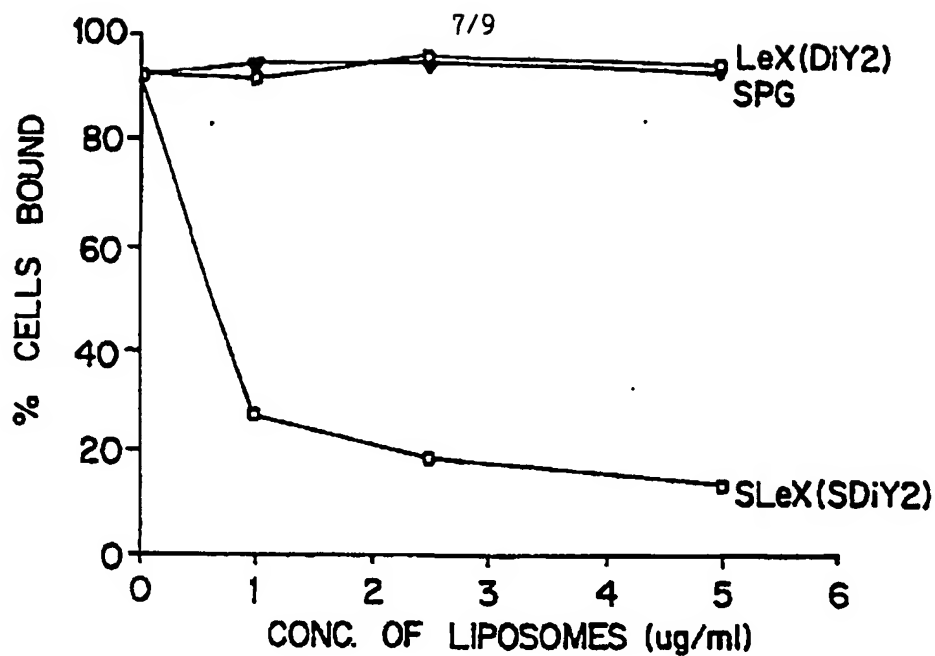
FIG. 8.

INHIBITION OF GMP-140 MEDIATED ADHESION
OF NEUTROPHILS BY GLYCOLIPID WITH TERMINAL
SIALIC ACID EITHER NeuAc OR NeuGc
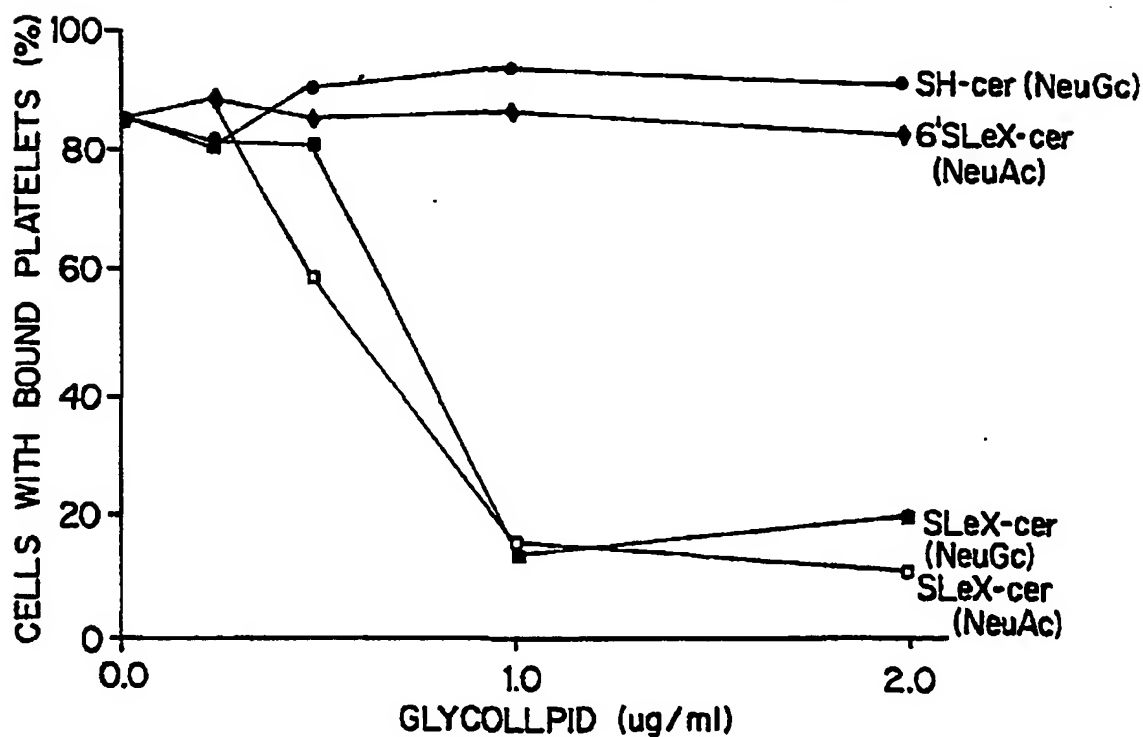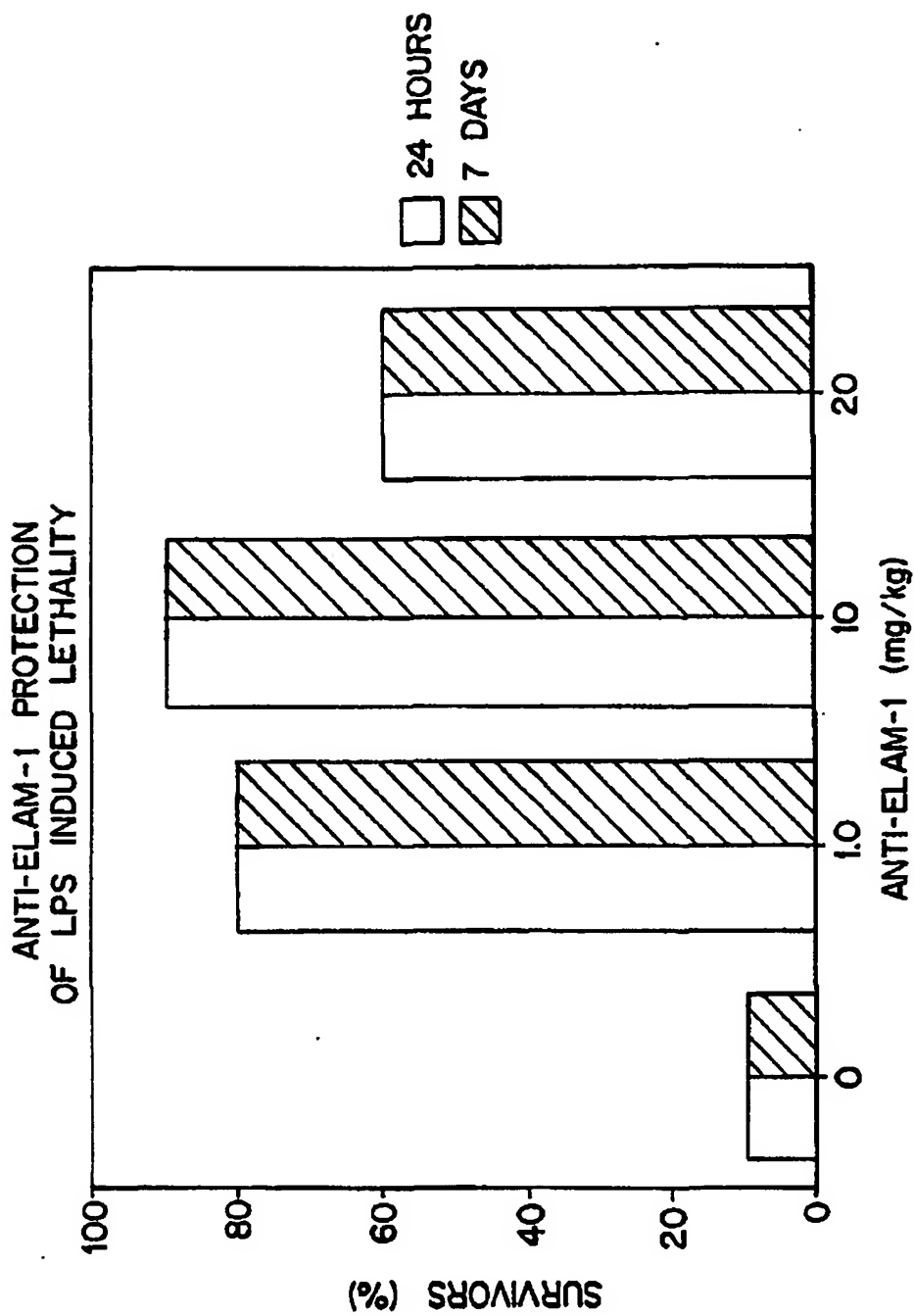
FIG. 9.

ANTI-ELAM-1 PROTECTION
OF LPS INDUCED LETHALITY

☐ 24 HOURS
▨ 7 DAYS

FIG. 10.

ANTI-ELAM-1 PROTECTION OF LPS
INDUCED LETHALITY IN RATS
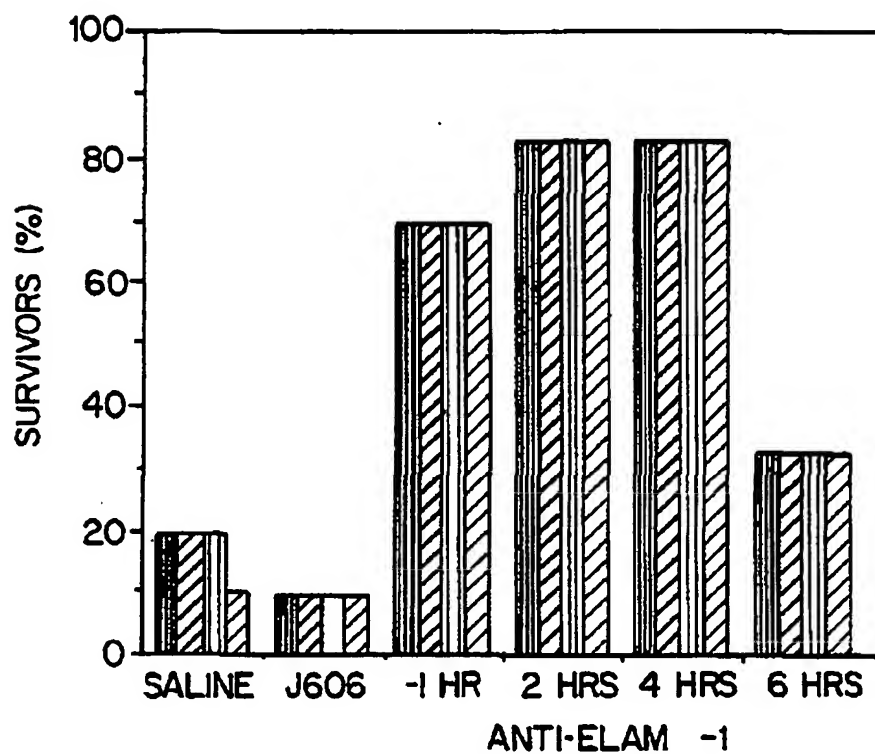
FIG. II.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, Indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 31/70, 37/02, 39/00, 37/20
U.S. CL.: 424/85.8, 450; 514/8, 23, 54; 536/1.1, 53, 123

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
U.S.	424/85.8, 450; 514/8.23, 54; 536/1.1, 53, 123

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

Chemical Abstracts Service: Selectin or ELAM and oligosaccharide(s) or polysaccharide(s) or saccharide(s) or carbohydrate(s)

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, [11] with Indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 259, no. 7, issued 10 April 1984, HAKOMORI ET AL., "Novel Fuco-lipids Accumulating in Human Adenocarcinoma", pages 4672-4680, see entire document.	1-81
Y.	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 259, no. 7, issued 10 April 1984, Y. FUKUSHI ET AL., "Novel Fuco-lipids Accumulating in Human Adenocarcinoma", pages 4681-4685, see entire document.	1-81
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 259, no. 16, issued 25 August 1984, Y. FUKUSHI ET AL. "Novel Fucolipids Accumulating in Human Adenocarcinoma", pages 10511-10517, see entire document.	1-81
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 260, no. 12, issued 25 June 1985, E.H. HOLMES ET AL., "Enzym-atic Basis for the Accumulation of Glycolipids with X and Dimeric X Determinants in Human Lung Cancer Cells (NCI-H69)", pages 7619-7627, see entire document.	1-81
	(cont.)	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21 September 1991	08 OCT 1991
International Searching Authority	Signature of Authorized Officer *Nancy S. Carson*
ISA/US	Nancy S. Carson (vsh)

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 263, no. 23, issued 15 August 1988, P. STANLEY ET AL. "The LEC11 Chinese Hamster Ovary Mutant Synthesizes N-Linked Carbohydrates Containing Sialylated, Fucosylated Lactosamine Units", pages 11374-11381, see entire document.	1-81
Y	SCIENCE, Vol. 243, issued 03 March 1989, M.P. BEVIL-ACQUA ET AL., "Endothelial Leukocyte Adhesion Molecule 1: An Inducible Receptor for Neutrophils Related to Complement Regulatory Proteins and Lectins," pages 1160-1165, see entire document.	1-81
Y,P	SCIENCE, Vol. 250, issued 23 November 1990, M.L. PHILLIPS ET AL., ELAM-1 Mediates Cell Adhesion by Recognition of a Carbohydrate Ligand, Sialyl-Le^X, pages 1130-1132, see entire document.	1-81
Y,P	SCIENCE, Vol. 250, issued 23 November 1990, G. WALZ ET AL., "Recognition by ELAM-1 of the Sialyl-Le^X, Determinant on Myeloid and Tumor Cells", pages 1132-1135, see entire document.	1-81
Y,P	NATURE, Vol. 346, issued 02 August 1990, T.A. SPRINGER, "Adhesion receptors of the immune system", pages 425-434, see entire document.	1-81
Y	CELL, Vol. 56, issued 24 March 1989, L.A. LASKY ET AL. "Cloning of Lymphocyte Homing Receptor Reveals a Lectin Domain", pages 1045-1055, see entire document.	1-81
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA), Vol. 87, issued March 1990, G. HESSION ET AL., "Endothelial leukocyte adhesion molecule 1: Direct expression cloning and functional interactions," pages 1673-1677, see entire document.	1-81
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 263, no. 20, issued 15 July 1988, K. DRICKAMER," Two Distinct Classes of Carbohydrate-recognition Domains in Animal Lectins," pages 9557-9560, see entire document.	1-81
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (USA), Vol. 84, issued December 1987, M.P. BEVILACQUA ET AL., "Identification of an inducible endothelial-leukocyte adhesion molecule," pages 9238-9242, see entire document.	1-81
Y,P	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, Vol. 112, No. 9, issued 1990, K.C. NICOLAOU ET AL., "Total Synthesis of the Tumor-Associated Le^X Family of Glycosphingolipids," pages 3693-3695, see entire document.	1-81